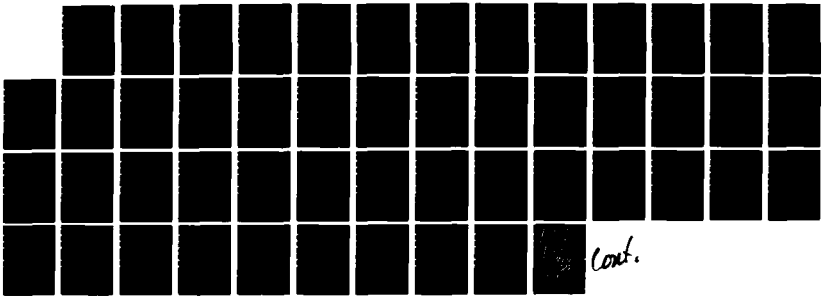
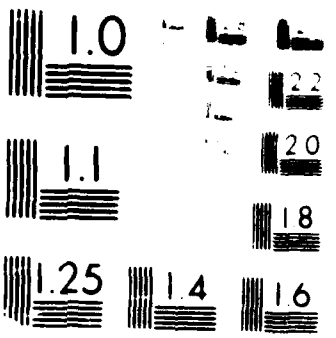
AD-A191 430 MECHANISMS OF RAPID NONSPECIFIC RESISTANCE INDUCED BY IMMUNOMODULATORS: D (U) MEDICAL COLL OF PENNSYLVANIA PHILADELPHIA DEPT OF MICROBIOLOG... P S MORAHAN ET AL. 1/2

UNCLASSIFIED 28 JAN 88 N00014-82-K-0669 F/G 6/5 NL



Cont.


1.0
1.1
1.25
1.4
2.2
2.0
1.8
1.6

Final Progress Report on Contract N00014-82-K-0669

Start Date: August 1, 1982.

1.0 Research objectives. The objectives have remained those of the original proposal and their extended developments which have been to:

1.1 Establish the effects of short term and long term treatment with ^{89}Sr on nonspecific effector cells in the mouse.

1.2 Establish the efficacy of selected immunomodulators on resistance to Listeria monocytogenes, EMC virus and HSV-2 in normal mice and in mice treated with the above regimens.

1.3 Extend developments of observations concerning:

1.31 Regulation of phenotypic expression of mononuclear phagocytic effectors.

1.32 The effect of the depletion of tissue MØ on host resistance.

2.0 Summary of progress in years 1-4.

2.1 Year 1. Experiments on the influence of ^{89}Sr administration to mice on effector cells dealt largely with short term (single dose) effects and early studies on long term (two dose) effects. Short term effects of bone marrow depression and leukopenia (with profound monocytopenia) resulted in the virtual elimination of monocyte elicitation by inflammatory agents and profound depression on splenic NK cell activity. Despite these drastic alterations to components of the host defense mechanism there was little effect on resistance to infection. In the long term treatment protocol, however, there was less of an effect on monocyte elicitation and resistance to Listeria was 100-fold reduced compared with the 3-9-fold reduction in the short term regimen. Treatment with C. parvum provided almost a 100-fold increase in resistance in both groups, however, based on growth of Listeria in the spleen and protection against mortality. Long term ^{89}Sr treatment also provided the depletion of mature NK cells.

The immunomodulatory effects of C. parvum, MVE-2, pyran copolymer, and the lipoidal amine, CP 20,961, were compared for protective activity against Listeria, and EMC and HSV-2 viruses in normal mice in preparation for experiments in ^{89}Sr treated mice.

2.2 Year 2. Studies begun in the first project year were extended and developed. The effects of acute and prolonged bone marrow ablation on the function and restoration or circulating monocytes, lymphocytes and PMN, peritoneal MØ, and splenic NK and NC cells were studied further. In the CD-1 mouse, treatment with ^{89}Sr, 4μCi/gram body weight (gbw), resulted in profound depression of all WBC, with no marked changes in peritoneal MØ and lymphocytes. There was, in

addition, a significant decrease in spontaneous as well as interferon (IFN)-inducible NK cell activity but no effect on splenic NC cell activity. Elicitation of PMN appeared less impaired whereas the elicitation of monocytes into the peritoneal cavity was virtually nil. There was evidence, however, for the activation of resident peritoneal MØ *in situ* by *C. parvum* or thioglycollate broth. In brief, the early period after ^{89}Sr treatment in the CD-1 mouse is characterized by a marked decrease of functions of both monocytes and NK cells with maintenance or an intact compartment of tissue MØ.

Long term (two dose) effects of ^{89}Sr maintained monocytopenia for over 50 days, and depressed spontaneous splenic NK activity for at least 35 days (longer if a second dose of ^{89}Sr is given). Moreover, ^{89}Sr completely eliminated the ability of splenic NK cells to be activated by IFN. A gradual decline in resident peritoneal MØ to 40-75% of control values was seen despite evident concurrent recovery of blood monocyte levels to normal.

Problems arose in connection with the specific activity of the supply of ^{89}Sr, first detected in unexpected and aberrant experimental results. The product originally employed was bought from Oak Ridge and was almost carrier-free but frequently had unacceptable levels of contamination with ^{90}Sr. The ^{89}Sr bought from Amersham Radiochemicals was very pure but gave irregular results which we found was due to the unusually low specific activity in some batches. Negotiations with the manufacturer led to their providing an assured specific activity of ^{89}Sr.

Extended studies on the effects of short term and long term regimens of ^{89}Sr on immunomodulation of effector cells showed that resident MØ could be activated for enhanced tumoricidal activity and the expression of characteristic ectoenzymes phenotypes. By 7 days after *C. parvum* treatment both spontaneous and IFN-inducible NK cell activity were reduced. These activities were even further reduced in ^{89}Sr treated mice. MVE-2 likewise appeared capable of activating resident peritoneal MØ *in situ*. PMN elicitation in MVE-2 treated mice was brisk in controls and diminished in ^{89}Sr mice in consonance with circulating levels of PMN.

2.3 Year 3. Effects of short term and long term ^{89}Sr leukocyte depletion regimens on natural resistance to *Listeria*, EMC virus and HSV-2 virus suggested that natural resistance to these agents does not depend on normally functioning circulating monocytes and NK cells. It was hypothesized that peritoneal and other tissue MØ populations may play an important role in preserving considerable resistance in ^{89}Sr treated mice.

Effects of short term and long term ^{89}Sr regimens on immunomodulator enhanced resistance showed that α-IFN appears to be more effective than ß-IFN against EMC virus. In short term ^{89}Sr treatment *C. parvum* induced resistance to Listeria was unaffected, a result comparable to earlier observations in the long term model.

A-1

Comparable results were obtained with other immunomodulators, MVE-2 and CP20-961 against HSV-2. Prophylactic treatment with MVE-2 one day prior to infection gave rise to over a 100-fold increase in resistance in normal mice and short term ^{89}Sr mice. This enhanced resistance was not associated with either increased spontaneous or IFN-induced NK cell activity. Additional studies with C. parvum in normal and ^{89}Sr mice showed protection against EMC virus in both short and long term ^{89}Sr protocols and in the controls.

Overall, the data support the concept that both natural and immunomodulator induced resistance to infection with a variety of microorganisms is independent of normal levels of circulating monocytes or NK cells. MHV infection impaired the quantitation of some experiments and the interpretation of additional experiments employing other modulators and interferons. We have subsequently ensured that all mice remain free of MHV infection and have documented the effect of MHV on MØ function and host resistance.

2.4 Year 4. A variety of immunomodulators were evaluated in normal mice for their ability to enhance nonspecific host resistance against HSV-2, EMC virus and Listeria monocytogenes. Several novel chemical immunomodulators with potent antiviral activity on prophylactic administration were identified; these included CL246,738, avridine in liposomes, and several pyrimidinones. In addition, repeated therapeutic administration of either α- or ß-IFN was effective, particularly against HSV-2 infection. Some results showed that treatment regimens effective against the viral infections may not be effective, or may even have adverse effects when employed against Listeria infection.

Work in the ^{89}Sr model of selective cellular depletion continued to demonstrate that tissue MØ populations are regulated independently of blood monocytes. The immunomodulator, C. parvum, has been very useful in this regard because it induces prostaglandin E_2 (PGE_2) releasing suppressor MØ in the spleens of normal mice but not in the macrophage-rich spleens of ^{89}Sr treated mice. Bone marrow function appears essential in the inductive mechanism but confirmatory experiments were elusive in view of the sustained radioactivity in the bone marrow of the ^{89}Sr mice.

The concept of tissue MØ independence was strengthened by observations in congenitally monocytopenic Sl/Sl^d mice. These mice showed conventional levels of resident peritoneal MØ but poor elicitation. On the other hand, PGE_2-releasing MØ were induced in abundance in their spleens following C. parvum treatment suggesting that these MØ, although apparently bone marrow dependent, are monocyte independent.

Data in resistance experiments continued to suggest that peritoneal MØ may be an important barrier to infection, at least when infectious agents are administered intraperitoneally. In response to these observations a series of experiments exploring the effects of

depletion of peritoneal MØ were begun. Advantage was taken of the ^{89}Sr model in which the circulating pool of monocytes is reduced below the capacity to emigrate in significant numbers when inflammatory agents are injected IP. Thus a model is provided for the evaluation of resident MØ. The approach to this problem was to study the feasibility of presentation of a cytotoxic agent selectively to peritoneal MØ. A system based on the encapsulation of ricin and ricin A-chain (RAC) in liposomes, representing particles for phagocytosis, became a subject of study. It became clear that despite an enthusiastic literature on liposomes as delivery systems, a large number of variables had to be dealt with before critical experiments could be undertaken. Problems addressed included the effects of liposome composition and structure, factors influencing uptake, effective measurements of cytotoxicity, efficiency of encapsulation of the aqueous phase and variations in cellular affinity for the liposomes. In vitro studies comparing such variables in resident and elicited MØ and in the MØ-like cell line, P388D1, were conducted. It was clear that additional groundwork had to be laid before reproducible and meaningful in vivo data could be expected.

3.0 Report of progress, Year 5.

3.1 The development of toxin-containing liposomes for the purpose of selective destruction of MØ. The purpose of these studies was to determine whether the selective elimination of a defined compartment of tissue MØ would result in diminished host resistance. It has been noted earlier that the near-elimination of blood monocytes with the bone-seeking isotope, ^{89}Sr, resulted in only a mild to moderate depression of host resistance (1). Since the infectious agents are initially instilled into the peritoneal cavity in such experiments, it seemed reasonable to focus on peritoneal MØ in monocyte-depleted mice. As before, mice would be depleted of monocytes by the iv administration of ^{89}Sr.

The approach chosen for this study was to deplete peritoneal MØ with the toxic plant lectin, ricin. The ricin in turn was to be encapsulated in liposomes, a particulate form, thereby selecting phagocytic cells. Earlier progress reports contain theoretical considerations and rationale which will be briefly summarized here. Ricin is a highly toxic glycoprotein with a LD_{50} in mice of 1.2g/100g body weight (2). It is isolated from castor beans (Ricinus communis), and when purified, has an approximate Mr of 65,000 (3). Isolation procedures from castor beans have yielded two major forms of ricin, the more toxic of which has been designated RCA II, and another, ricin agglutinin, RCA I (Mr 120,000) (3). RCA II is a heterodimer composed of a 32 kD A chain and a 34 kD B chain joined by an S-S bond (4). When tested separately, only the A-chain proved toxic (5) whereas the capacity to bind the cell surface receptors is associated with the B-chain (5). The mechanism of toxicity is thought to be inhibition of protein synthesis by inactivation of the 60S ribosomal subunits by A-chains acting in enzymatic fashion (6). During the

course of the project information published by others indicated that the binding of whole ricin to MØ is in fact a selective process dependent on mannose receptors expressed on these cells (7). Pursuit of effective liposomal delivery of ricin was considered important, however, to minimize both the inflammatory effects of free ricin in the peritoneal cavity and its systemic toxicity.

3.11 Preparation of Liposomes. In most experiments negatively charged multilamellar liposomes were prepared from phosphatidyl choline (PC), phosphatidic acid (PA) and cholesterol in a molar ratio of 7:1:3. The lipids were dissolved in chloroform, evaporated under a stream of nitrogen and dispersed in 0.005 M phosphate buffer, pH 7.2 in 0.2 M NaCl employing an ultrasonic bath.

Unilamellar liposomes were prepared by solvent exchange in a multichambered rotary dialysis apparatus (Lipoprep^R, DIANORM-Gerate, Munich, West Germany) with lipids initially dissolved in a glucopyranoside solution.

The interiorization or ricin and other proteins was evaluated by nuclear spin resonance as described below. Less stringent methods of estimating liposome-associated protein included the use of isotopically-labeled ricin and amino acid analysis. Unincorporated or unbound protein was removed by washing/ultracentrifugation at 45,000 - 100,000 xg and removal of supernatants or by passage of liposomes through a Sephadex G200 column.

Estimates of lipid uptake were made by means of ^{3}H-cholesterol added to the lipid mix before solvent evaporation.

3.12 Nuclear Magnetic Resonance Studies. In using liposomes to deliver ricin to cells, it is important to know the location of the ricin in the liposome, i.e., whether it is located in the interior solution or in the lipid bilayer region itself. If the ricin is located in the lipid bilayer, it then becomes of interest to know whether it interacts with the polar head-groups of the liposome or with the hydrophobic interior of the bilayer. The interaction of ricin with its liposome carrier could well affect the interaction of the liposomes with cells and, therefore, the delivery of the ricin to the cells. One of the best ways of determining the nature of the ricin-liposome interaction is by the use of nuclear magnetic resonance (NMR) techniques. The same liposome encapsulated ricin samples which were used for the cell studies were prepared in deuterated water so that the proton NMR could be accomplished. Proton NMR spectra (400 MHZ) were obtained at the NSF sponsored NMR laboratory at the University of South Carolina, Columbia, South Carolina. Shown below (Fig. 1) is a proton NMR spectrum of the liposomes containing ricin. We find that there is little difference between this spectrum and one from the same liposome without ricin, thus suggesting that if ricin interacts with the liposome the interaction is a rather weak one. It seems reasonable to conclude that the ricin is present in the core solution of the liposome and in

the solution intercalated between the bilayers of multilayer liposomes. This would mean that the interaction between the cells and liposomes depends on the lipid composition of the vessels with the ricin having little effect on this cellular interaction. The liposomes thus act only as carriers for the ricin and presumably release it into the cell after the liposome cell interaction.

Carbon-13 and phosphorus-31 spectra of the liposome encapsulated ricin were also obtained and compared with NMR spectra from control liposomes. The phosphorus spectra did show differences between the control and the ricin liposomes, when the liposomes were exposed to chaotropic anions, a common test for liposome structure. Further study would be needed in order to determine whether this was an indication of a ricin lipid head-group interaction. Additionally, the proton NMR head-group signal is unaffected by the chaotropic anion when ricin is present. Again, whether this is suggestive of a ricin lipid head-group interaction or merely scavenging of the anion by free ricin remains to be determined.

On the basis of our results, it appears that ricin is located primarily in the solution contained within the liposomes rather than in the hydrophobic interior of the lipid bilayers. Some ricin interaction with the lipid head-group cannot be ruled out, but would not be expected to affect the cell-liposome interaction.

3.13 Toxicity studies in vitro with liposome containing toxins. Ricin A-chain was encapsulated in liposomes and incubated in doses of 0-40 ng/ml with peritoneal MØ isolated by adherence to plastic dishes for 3 hours. After washing away the liposomes, the ^{3}H-leu incorporation was found to be decreased by only about 20% below control levels, suggesting a low uptake of liposomes by resident MØ. Dose dependence was not clear cut at the low levels of ^{3}H-leu incorporation observed in this experiment.

A troublesome and recurrent feature was the evident toxicity of "empty" multilamellar liposomes for MØ, which sometimes resulted in a 60-65% reduction of ^{3}H-leu uptake compared with MØ in medium without liposomes. MØ incubated in vitro with A-chain liposomes showed an additional 20-30% reduction. The toxic property associated with liposomes prepared by sonication was effectively reduced by dialysis; unilamellar liposomes prepared in the Lipoprep (see Sect. 3.11) apparatus showed little toxicity. These observations are summarized in Table 1.

Although the samples were counted at low levels of radioactivity, it is clear that differences between dialyzed and undialyzed samples are highly significant. The medium control, MØ in RPMI, with no liposomes, however, yielded still higher viability and leucine uptake than the best preparations. The data also show that partially purified PC ("crude") has an apparent effect on viability whereas the other preparations show a dissociation between viability and ^{3}H-leu uptake.

The data suggest that the apparent toxicity associated with undialyzed liposome preparations may adversely affect protein synthesis in MØ, and is thus a potential source of artifact in the evaluation of ricin toxicity. It is possible that low molecular weight contaminants of egg yolk origin be the source of cytotoxicity rather than the persistence of residua of solvents employed in the preparation of the liposomes. As a result of these observations, most of the subsequent protocols employed multilamellar liposomes prepared by sonication, purified PC, and followed by dialysis, washing, or passage through a Sephadex G200 column.

Figure 2 illustrates that the uptake of ^{3}H-cholesterol labeled multilamellar liposomes by thioglycollate elicited MØ and P388D1 cells is comparable and approximately two-fold higher than uptake by resident peritoneal MØ (RPM). <Microporous filtration to achieve increased homogeneity of liposomal size resulted in a 25-30% reduction in liposomal uptake by RPM and P388D1 cells but uptake by exudate MØ was unaffected. Because liposomes were in excess in all the preparations, this observation that RPM an P388D1 cells take up small liposomes less effectively than large liposomes, whereas exudate MØ take up the whole range of liposomes equally well.

The synthesis of protein by the 3 types of MØ (see preceding paragraph) was monitored by the incorporation of ^{3}H-leucine (^{3}H-leu) in vitro. Figure 3 shows that uptake by RPM is relatively low compared with ^{3}H-leu uptake by exudate MØ and P388D1 cells. The differences are shown more strikingly when these 3 classes of MØ are incubated with ^{3}H-leu for longer intervals (Figure 4). Since the differences between the cells of primary interest, RPM and exudate MØ, are already highly significant after a 1 hr incubation, however, this convenient interval was employed in most of the subsequent in vitro studies.

It was considered that resident MØ in ^{89}Sr treated mice would respond to IP stimuli and act like exudate MØ, at least as far as the expression of ectoenzymes is concerned (8). This is a sound interpretation since there is usually little or no MØ exudation in such mice which have profound monocytopenia consequent to bone marrow ablation by ^{89}Sr (8). On the other hand , it was thought possible that protein synthesis by stimulated resident peritoneal MØ in ^{89}Sr treated mice by agents such as Listeria, would not increase greatly. Damage inflicted by the incorporation of the toxic ricin-liposomes might thus be difficult to assess. Attempts were therefore made to find more sensitive assays of cellular damage than ^{3}H-leu uptake, an index of protein synthesis, and trypan blue exclusion, a gross index of viability. Release of 1-^{14}C-amino-isobutyric acid (AIB) was tried as recommended by Thelestam and Mölby (9). The procedure is analogous to ^{51}Cr release but is supposed to be a more sensitive indicator of early membrane damage (9). Amphotericin B was employed as a generic cytotoxin in some of these experiments to permit comparison of our data with results in the literature. In our hands, however,

spontaneous release of AIB proved too high to provide a really sensitive assay. A 90 minute ^{51}Cr release assay, on the other hand, proved very reliable for the assessment of the toxic effect of 3-5 x 10^{-5}M amphotericin B on unstimulated mouse peritoneal MØ in vitro (Fig. 5).

An additional study investigated the use of previously incorporated ^{3}H-leu in a release assay employing Amphotericin B (1-3 x 10^{-5}M) as the toxic agent (Table 2). Resident MØ, thioglycollate elicited (4 days) MØ and the MØ-like cell line, P388D1 were compared. Toxicity was evaluated as with ^{51}Cr:

$$\% \text{ Toxicity} = \frac{\text{Toxic release - spontaneous release}}{\text{Maximal release - spontaneous release}} \times 100$$

Table 2. shows that the response takes place in an approximately dose dependent manner with comparable toxicities observed at the higher dose of Amphotericin B. The reason for the variability at the lower dose is uncertain, but the data suggest the possibility that differences in resistance to the toxic effects of amphotericin B at low dosage are overcome at higher dosages.

Although some of these assays appeared useful they were reserved in favor of ^{3}H-leu uptake since the toxic action of ricin is expressed as impaired protein synthesis.

3.14 Evaluation of Ricin Toxicity. A recent report by Simmons et al (7) showed that unencapsulated ricin binds selectively to murine peritoneal MØ, apparently through a mannose receptor. It was therefore decided to compare the effects on peritoneal MØ of unencapsulated ricin with ricin and ricin A chain administered in multilamellar liposomes. When P388D1 cells were used as a model population in vitro, nearly maximal effects were seen as early as 3 hrs of incubation with as low a concentration of ricin as 0.25µg/ml (Fig. 6). The initial results, however, were distressingly variable with respect to the uptake of ^{3}H-leu. It was found that optimal uptake required still further adjustment of the medium and that maximal uptake, as high as 18% of available radioactivity, was achieved in RPMI 1640 diluted 1:1 with Ca^{++} and Mg^{++} free PBS although conventional complete RPMI 1640 provides results well up on the curve (12% uptake) compared with incubation in PBS alone (0.2%) or in Hank's balanced salt solution (2%).

An in vitro dose response of MØ to ricin and ricin A chain (RAC) showed that RAC is indeed directly toxic, but that complete ricin is about 2-3 times as toxic as equal weights (Fig. 7) and thus approximately 4-6 times more toxic when calculated on an equimolar basis. Both whole ricin and RAC appear to be at maximal toxicities

for resident peritoneal MØ at the lowest doses employed (Fig 7A); protein synthesis in P388D1 cells appears to be more resistant, particularly to RAC (Fig 7B). Studies in vivo, however, showed that mice which received free RAC in log incremental doses of 0.01 to 10µg ip (0.025 - 25µg/ml), displayed no signs of toxicity and that peritoneal MØ were depleted only by whole ricin (Fig. 8). The observed difference suggests that RAC may inactivated when given in vivo. Only 1µg (2.5µg/ml) of whole free ricin IP, on the other hand, effected a 90% reduction in peritoneal MØ in 18 h. Figure 8 also shows that toxicity increased sharply from the preceding lower dose of 0.25µg/ml which appeared to have no effect. The next higher dose of 10µg is assumed to have been uniformly lethal since the lavage specimens were obtained from mice which were clearly moribund. A dose response analysis of 4 dilutions from 0.1 to 1.6µg (.25-4µg/ml) of free ricin IP showed definite dose dependence (Fig 9). It is interesting that these mice showed a peak increase in PMN at 0.1µg of ricin which was sustained to 0.8µg then fell off sharply at 1.6µg suggesting that the influx of PMN was unaffected until a sufficient peritoneal concentration of ricin was reached. Taken further, the observation suggests that receptors for ricin may be present on PMN cell membranes but possibly the binding affinity is low. Results, however, were inconsistent from experiment to experiment. Lymphocyte numbers were totally unaffected at all doses of ricin, strongly suggesting that no receptors for ricin are present on these cells. Data from these studies on free ricin provide a comparison for the assessment of the relative merits of liposomal packaging and delivery. It was not pursued for its own sake because of the high toxicity and inflammatory potential of free ricin.

3.15 Depletion of mouse peritoneal MØ with silica was attempted with two different products, Min-U-Sil (Whitaker, Clark and Daniels, Plainfield, N.J.) and Doerentrupper Silica #12. The dose employed was 0.5ml of a 100 mg/ml suspension in PBS. Although MØ numbers were 25-27% below normal levels at 24 hrs the standard deviations were large with some mice showing no significant decreases. By 48 hrs, MØ were in excess of normal, and values returned to normal by 96 hrs. It was concluded that the "window" of depletion achieved by this method was too narrow and that multiple doses of silica would be necessary. Each dose injected would thus impose added artifacts including the cumulative presence of larger numbers of particles than occurs with liposomes since the silica is not metabolized. The effects of silica were disappointing; it was decided not to proceed with silica, but to focus on liposome encapsulated toxins.

3.16 Depletion of mouse peritoneal MØ with liposome encapsulated toxins. Ricin and RAC were incorporated into negatively charged multilamellar liposomes prepared as PC:PA:cholesterol in a molar ratio of 7:1:3 as described earlier, by sonication in a bath. Unincorporated toxin was removed by ultracentrifugation at 45,000 - 100,000 xg and decantation repeated twice. Fig. 10 shows that ricin-liposomes reduced the number of peritoneal MØ by 74% in 18 h, whereas RAC had no more effect than empty liposomes.

An additional toxin, dichloromethylene diphosphonate (DMDP) was also tested for MØ in view of the recent reports in the literature by van Rooijen and van Niewegen (10). This reagent, kindly provided by Dr. E. Cabrero, Norwich Pharmaceutical Corp., was encapsulated in multilamellar liposomes as described by van Rooijen and van Niewegen (10). In contrast to the liposomes employed in most of our studies (see preceding paragraph), these liposomes are prepared without PA and hence carry little or no charge. In brief, 75 mg PC and 11 mg of cholesterol were dissolved in chloroform, evaporated under vacuum, and 10ml of 10mM PBS containing 1.89g of DMDP added. After 2h standing, the mixture was dispersed in a bath-type sonicator at 50Hz. After 2 additional hours of standing, the liposomes were washed by centrifugation at 100,000 xg at 30° C. The liposome pellet was resuspended in 4ml PBS. Empty liposomes were prepared in a like manner without the addition of DMDP. Mice were treated by ip injection of 0.2ml of either non-encapsulated DMDP, encapsulated DMDP or empty liposomes. Cells were harvested 24 h later. Mice given 0.2 ml of free DMDP (1.89g/10ml), died within 5 min of injection. Figure 11 shows that by 24 h after injection, empty liposomes had not provoked any significant changes among major cell classes, but DMDP liposomes resulted in a 30% reduction in MØ accompanied by a large exudate of PMN. Although the difference in MØ numbers between the PBS controls and the DMDP liposome treated mice was not statistically significant, the differences between the DMDP/liposome and empty liposome control showed a significant reduction in MØ and increase PMN, $p < 0.01$ (Student's t test). Additional and extensive experiments would be required to assess the significance of the differences of the results obtained with ricin-and DMDP liposomes. Subsequent studies on peritoneal MØ were therefore focussed predomintly on ricin-liposomes although DMDP-liposomes were included in some protocols.

3.17 Studies with IP liposome-toxins in monocyte-depleted mice. A comparative study was performed in mice depleted of blood monocytes and bone marrow by the iv administration of ^{89}Sr, 2μCi per gram body weight on day zero. Controls received ^{88}Sr equivalent to the amounts of carrier in the radioactive inoculum. Groups of ^{89}Sr and control mice were each further divided into panels of 20 for each IP treatment: normal saline, empty liposomes, free ricin, ricin in liposomes, RAC in liposomes and DMDP in liposomes, each prepared according to earlier protocols. Ten days following Sr administration 0.4ml of each of the preparations listed above was administered to each mouse. Mice were sacrificed in groups of 5 on days 10, 13, 24 and 27. The results are shown in Fig. 12 a-e.

In Fig 12, the data show that control ranges were established for blood monocytes and peritoneal MØ from the data for saline injected mice. The most effective depression of peritoneal MØ, about 80%, was seen in the free ricin group which survived for only 4 days. Treatments with RAC-liposomes and ricin-liposomes were highly effective in depressing peritoneal MØ for only one day although

residual effects were seen throughout the experiment. That is, high numbers of MØ suggestive of cellular exudates were seen in ^{88}Sr mice treated with RAC-liposomes and ricin-liposomes. It is interesting that the ricin and RAC liposome treated ^{89}Sr mice showed evidence of elicitation, although the numbers of cells were lower than those found after IP thioglycollate broth. The numbers of MØ in the corresponding ^{89}Sr mice were also always above control levels in the RAC-liposome group from days 13 to 27. In the ricin-liposome group, MØ in excess of normal numbers were seen only on day 13.

Data for monocytes and MØ in the DMDP-liposome treated group shown in Fig 12, is difficult to interpret), but exudation was pronounced as judged by the numbers of cells. The only exceptions appeared to be in the day 13 ^{89}Sr group and the 24 ^{88}Sr controls. Overall, DMDP liposome treatment appears to reverse monocyte depletion although the data show significant monocyte depletion on days 13 and 24. The day 10 data for blood monocytes show wide variances, the reason for which is unclear. Although such data would appear to disqualify DMDP-liposomes as an effective means of peritoneal MØ depletion, it would be interesting to determine the source of the elicited peritoneal cells in the ^{89}Sr mice. Further study, beyond the scope of the present investigations, would be required for this information.

In aggregate, the data show that complete ricin-liposomes injected IP into ^{89}Sr mice provide a narrow window which may be sufficient to test the hypothesis concerning the potentially protective role of peritoneal MØ when monocyte-depleted mice are infected IP.

3.18 The effect of IP ricin liposomes on resistance to infection with Listeria monocytogenes was studied in additional experiments. CD-1 female mice were divided into test (^{89}Sr) and control (^{88}Sr) groups, then further subdivided into treatment groups which received intraperitoneal liposomes, with and without encapsulated ricin. Still further subdivision was made into Listeria dosage groups spanning dilutions of freshly cultured organisms from 10^{-3} to 10^{-6}; 10^{-3} represented approximately one LD_{50}, containing 3.5×10^5 CFU of L. monocytogenes, strain EGD. Liposomes were prepared as before with PC:PA:cholesterol in a molar ratio of 7:1:3 and a ricin concentration of 2.5 mg/ml. Survival, microbial growth, blood monocyte levels and peritoneal MØ counts were monitored. The control for the effectiveness of the batch of ^{89}Sr to deplete monocytes was found to yield 87% depletion of blood monocytes 13 days after isotope administration.

In this study, the data were consonant with our previous observation (1) that there was no major difference in natural resistance between ^{89}Sr and ^{88}Sr treated mice to IP infection with Listeria. Treatment with ricin-liposomes, however, in both the ^{88}Sr and ^{89}Sr treated mice, caused a profound decrease in resistance. Fig. 13 and Table 3 show that there was little difference in survival between the ^{89}Sr and ^{88}Sr mice that received ricin liposomes: all mice died by day 9 after infection with roughly the same kinetics of

the survival curves. Mice which did not receive ricin-liposomes showed about a 50% survival for over 2 weeks irrespective of ^{89}Sr administration (Fig. 13b). Therefore, IP administration of ricin liposome caused a profound decrease in resistance to IP infection with Listeria, regardless of whether the mice were treated with ^{88}Sr or ^{89}Sr.

Representative mice were sacrificed 48 h after infection and cultures prepared from spleen, liver and peritoneal cells. The results summarized in Table 3 indicate that the death due to Listeria infection was related to increased growth of bacteria in the livers, spleens and peritoneal cavity. In mice treated with ^{88}Sr and ricin-liposomes, there was a 135- fold increase in the growth of Listeria. There was also a marked increase, although less, in the mice treated with ^{89}Sr and ricin-liposomes.

In a repeated experiment, there was also a marked increase in susceptibility among the mice receiving ricin-liposomes as evidenced by the survival curves (Fig 14) and the increased growth of Listeria in the organs of all mice treated with ricin liposomes (Table 4). An unexpected occurence in this experiment was that there were no deaths in the empty liposome ^{88}Sr group (data not shown) and only a few deaths in the empty liposome ^{89}Sr group. There is no apparent explanation for these occurrences at this time since the administered doses of Listeria were quantited by plate counts and little time had elapsed since determination of the LD_{50}.

Monocyte counts were problematic because the ^{89}Sr controls showed no depletion (Fig 15a), an unusual occurrence. This observation is all the more puzzling because by 48 h after infection (13 days after ^{88}Sr or ^{89}Sr) there appeared to be significant monocyte depletion in most of the surviving ^{89}Sr treated mice, but unexplained depletions in some of the ^{88}Sr mice (Fig 15a). The observed irregularities may also account for some of the variations in the Listeria growth studies. Peritoneal MØ counts 48 hrs after infection were widely distributed with a tendency to lower counts amounts among the ricin/liposome treated mice (Fig 15b). In the empty liposome panels lower values were found among ^{89}Sr mice than in the ^{88}Sr controls.

3.19 The effect of IV DMDP liposomes on resistance to infection with Listeria and HSV-2. In addition to evaluating selective depletion of peritoneal MØ and the subsequent effect on host resistance, we have been evaluating the effect of selective depletion of splenic and liver MØ by IV treatment of CD-1 mice with liposomes encapsulating the toxic dichloromethylene diphosphonate (DMDP). This material, when injected IV, has been reported to have profound depleting effects on splenic and liver MØ for at least one week (10-12).

When we inoculated CD-1 mice twice IV (days -4 and -2 prior to assay with saline, free liposomes or DMDP liposomes, there was no change in peritoneal MØ as evidenced by number, differential or

ectoenzyme patterns (Table 5). Cytochemistry on frozen spleen sections for the presence of acid phosphatase and the F4/80 MØ antigen confirmed the loss of MØ in the spleens of DMDP liposome treated mice (unpublished data in collaboration with van Rooijen). Treatment with DMDP liposomes did cause a marked leukocytosis in the blood, primarily of lymphocytes and PMN (Table 6). Interestingly, we also found a marked decrease in splenic spontaneous or interferon inducible NK cell activity: the activity returns to normal within 3 days (data not shown).

Several experiments have been performed to assess the effects of DMDP liposomes on host resistance to HSV-2 infections in the CD-1 mouse. No significant effects on resistance was observed when mice were treated with a single dose of DMDP liposomes 2 days prior to IV infection with HSV-2 (Table 7). The data in Table 5 suggest that single treatment with DMDP liposomes may not be sufficient, because there was no effect on circulating leukocytes or NK cell activity. Double IV treatment with DMDP liposomes, however, caused a profound decrease in resistance to IV infection with HSV-2: most mice died down through the 10^{-5} challenge dose which contained only 28 PFU (Table 7). In contrast to this profound effect on IV infection with HSV-2, there was only a mild effect on IP infection with HSV-2 (Table 7). The double IV treatment of mice with DMDP liposomes caused a slight decrease in natural resistance overall when the data were analyzed by Cox's proportional hazards general linear model ($p < 0.05$). There was no difference in LD_{50}, however, and only a slight decrease in MST in some groups.

The effect of two IV injections of DMDP liposomes on host resistance to IV infection with Listeria has also been assessed. DMDP liposome treatment markedly decreased resistance to Listeria as evidenced by increased mortality, decreased survival time, and a reduction in the number of bacteria required to produce an LD_{50} (Table 8). The number of CFU required to produce an LD_{50}, which was 1.2×10^5 CFU/LD_{50} in the saline and empty liposome group, was reduced to $< 2.3 \times 10^4$ CFU/LD_{50} for the DMDP liposome group. Treatment with DMDP liposomes also significantly reduced the median survival time of mice to 2.5 days.

These data showing none or very modest changes in IP infection or on the peritoneal MØ population after IV DMDP liposomes, which cannot penetrate the capillaries and gain entrance into the peritoneum, provide additional support for the concept that peritoneal MØ are maintained separately from at least several other MØ compartments in the body. We believe that the depressed host resistance observed after iv administration of DMDP liposomes and iv infection is most probably related to toxic effects on NK cells. However, we have previously demonstrated (1), and this report, that a decrease in NK cell activity in the CD-1 mouse does not markedly affect host resistance to IP infection with Listeria or HSV-2.

Table 1. The Effect of Dialysis on Liposomal Toxicity

Liposome composition	Dialyzed	% Viable[a]	CPM[b]
PC (crude), PA, cholesterol	+	ND	40
PC (crude), PA, cholesterol	-	22	89
PC (pure), PA, cholesterol	+	80	432
PC (pure), PA, cholesterol	-	86	186
PC (crude), PA, cholesterol	"Lipoprep"	80	445
Medium control	-	96	707

a. Trypan blue exclusion.
b. Means of duplicate counts.

Unstimulated Mø were obtained from CD-1 mice by peritoneal lavage with Ca^{++} and Mg^{++} free PBS and isolated by adherence to plastic petri dishes overnight, approximately 10^6/plate. Liposomes and Mø were incubated together for 3 hr, after vigorous washing, ^{3}H-leucine or trypan blue were added for 1 hr and 3 minutes, respectively. Cells incubated with ^{3}H-leu were washed then lysed with 0.1% triton X-100 @ 37° for 30 min, suspended in scintillant and the radioactivity counted in a liquid scintillation counter.

Table 2. Evaluation of Cytotoxicity by ^{3}H-leucine Release.

	Amphotericin B Concentration	
	1×10^{-5} M	3×10^{-5} M
Resident Mø	30.3[a]	46.6
Exudate Mø[b]	14.0	42.0
P388D1	11.1	42.0

a. Net % toxicity.
b. Four day thioglycollate elicited Mø.

Mø prelabeled with ^{3}H-leu were incubated in amphotericin B and vehicle for 90 minutes. Three freeze-thaw cycles were employed to lyse cells for maximal release of the isotopic label from untreated cells. Spontaneous release was measured as radioactivity in the culture supernatants of untreated unlysed cells. Toxic release was measured as radioactivity in the culture supernatants of cells incubated with a specified cytotoxic agent, in this case, amphotericin B.

Table 3. Effects of Ricin-Liposomes (i.p.) on Resistance to *Listeria monocytogenes* i.p. in ^{88}Sr or ^{89}Sr Treated Mice

Group		Mortality Analysis			Listeria Titers 48 hr. p.i. as log_{10} CFU Av. of 2 mice (log_{10} difference from control)					
Lipo-Ricin	^{89}Sr	Dose (CFU)	Dead/ Total	MST	Spleen		Liver		Peritoneal Cavity	
+	-	$3.8x10^5$	10/10	4.0	6.8		6.2		4.1	
+	+	$3.8x10^5$	10/10	3.8	6.0		6.5		4.6	
-	+	$3.8x10^4$	2/10	>10.0	4.5		2.4		<1.7	
+	-	$3.8x10^4$	10/10	3.4	5.7	(1.2)	6.5	(4.1)	4.5	(2.8)
-	+	$3.8x10^4$	4/10	>10.0	3.3		4.1		<1.7	
+	+	$3.8x10^4$	10/10	3.0	4.1	(0.8)	3.2	(0)	1.7	(0)
-	-	$3.8x10^3$	2/10	>10.0	2.0		<2.0		1.4	
+	-	$3.8x10^3$	10/10	3.4	5.5	(3.5)	5.6	(3.6)	4.0	(2.6)
-	+	$3.8x10^3$	4/10	>10.0	3.6		2.9		<1.7	
+	+	$3.8x10^3$	9/10	3.5	3.4	(0)	3.7	(0.8)	2.7	(1.0)
-	-	$3.8x10^2$	2/9	>10.0	<2.0		<2.0		<1.7	
+	-	$3.8x10^2$	9/10	3.6	3.5	(1.5)	3.8	(1.8)	3.3	(1.6)
-	+	$3.8x10^2$	3/10	>10.0	2.8		<2.0		2.2	
+	+	$3.8x10^2$	10/10	3.7	4.7	(1.9)	4.4	(2.4)	2.9	(0.6)

Table 4. Effect of i.p. Administration of Ricin-Liposomes on Resistance of Mice to i.p. Infection with *Listeria monocytogenes*[a]

Treatment		Log10 CFU Listeria in		
89Sr	R-L	Spleen	Liver	Peritoneum
-	-	1.6 ± 0.4	<1.0 ± 0	<0.7 ± 0
-	+	3.2 ± 1.5 (1.6)[b]	2.9 ± 1.0 (>2.9)	2.7 ± 1.0 (>2.0)
+	-	1.5 ± 0.4	1.7 ± 0.7	0.9 ± 0.1
+	+	4.4 ± 0.3 (2.9)	4.3 ± 0.5 (2.6)	3.3 ± 0.1 (2.4)

a CD1 female mice were injected i.v. with ^{88}Sr or ^{89}Sr, and i.p. with free liposomes or ricin liposomes (R-L) 13 days later. The next day mice were challenged with approximately an LD_{50} dose of Listeria, and were sacrified 28 hours later. Spleens and livers were obtained, weighed, frozen at -70°C, homogenized and the titer of Listeria per organ determined by colony formation (CFU) on blood agar. The peritoneal compartment was lavaged with 5ml of PBS, frozen and the titer of Listeria per 0.5 ml determined. Each group consisted of 3-5 mice.

b Numbers in parenthesis = $\log_{10}$ difference in geometric means of the R-L groups compared with the respective control free lipsomes groups.

Table 5. Lack of Effect of i.v. DMDP Liposomes on Peritoneal Cells

Exp. Group	Exp. #	PEC/Mouse ($x10^6$) MØ	PEC/Mouse ($x10^6$) LY	Ectoenzyme SA 5'N	Ectoenzyme SA APD
Naive	1	3.9 ± 0.2	1.5 ± 0.03	10.2 ± 3.0	19.4 ± 1.1
	2	1.8 ± 0.1	3.1 ± 0.5	11.7 ± 7.0	16.0 ± 1.8
NaCl	1	3.5 ± 0.5	1.6 ± 0.3	15.2 ± 5.3	22.7 ± 2.0
	2	2.0 ± 0.3	3.1 ± 0.4	10.1 ± 1.3	20.2 ± 1.9
Free Lip	1	4.1 ± 0.3	1.3 ± 0.2	14.0 ± 2.7	18.6 ± 0.5
-3, -1	2	ND	ND	ND	ND
DMDP Lip	1	3.9 ± 0.6	1.6 ± 0.3	12.7 ± 1.9	19.1 ± 1.7
-3, -1	2	2.0 ± 0.5	2.6 ± 0.2	8.6 ± 1.4	19.4 ± 2.5
DMDP Lip	1	ND	ND	ND	ND
-2	2	1.9 ± 0.3	2.8 ± 0.2	8.2 ± 1.4	20.4 ± 0.8

There were no significant differences in any groups as compared with naive or NaCl control groups.

Table 6. Effect of DMDP Liposomes on WBC, PEC and NK cells

Exp. Group	Exp. #	WBC/ml (#x10^5) ± SE			NK cell Cytotoxicity (% ± S.E. at 200:1)	
		Mono	Ly	PMN	Spontaneous	+ IFN
Naive	1	2.0 ± 0.7	60.0 ± 6.7	17.1 ± 3.5	15.0 ± 3.6	40.1 ± 5.7
	2	ND	ND	ND	22.1 ± 4.2	50.5 ± 4.3
NaCl	1	6.0 ± 1.2	58.0 ± 10.1	17.0 ± 2.3	21.6 ± 1.9	51.0 ± 3.9
	2	2.5 ± 0.6	43.2 ± 4.4	8.6 ± 1.1	31.5 ± 1.0	53.2 ± 2.1
Free Lip. -3, -1	1	3.8 ± 1.0	54.0 ± 7.7	32.3 ± 8.8	19.5 ± 2.2	46.6 ± 3.0
	d	ND	ND	ND	ND	ND
DMDP Lip. -3, -1	1	5.2 ± 0.9	136.0 ± 13.7*	49.7 ± 9.6*	3.0 ± 0.5*	10.6 ± 2.9*
	2	6.2 ± 1.4*	107.6 ± 15.3*	32.0 ± 13.6*	6.2 ± 1.9*	17.2 ± 3.8*
DMDP Lip. -2	1	ND	ND	ND	ND	ND
	2	1.5 ± 0.5	62.4 ± 8.2	19.2 ± 8.3	7.3 ± 2.6	26.5 ± 4.6*

* $p < 0.05$ by ANOVA and Tukey post hoc test as compared with naive group for all data in Exp. 1 and 2, except for comparison with the NaCl group for WBC data for Exp. 2.

Table 7. Effect of i.v. DMDP Liposomes on Resistance to HSV-2 Infection in CD-1 Mice

Virus Dilutions		Saline	Free Liposomes	DMDP Lip. Day-2	DMDP Lip. Day-3, -1
Exp. 1 HSV-2 i.p. infection					
-1.0	% Dead	80.0	100.0	100.0	100.0
	MST	11.0	7.8	7.8	8.0
-1.5	% Dead	80.0	100.0	100.0	100.0
	MST	11.2	7.8	9.2	8.0
-2.0	% Dead	80.0	80.0	100.0	100.0
	MST	12.8	11.8	7.0	9.0
-2.5	% Dead	100.0	100.0	100.0	100.0
	MST	10.0	11.8	9.8	9.2
-3.0	% Dead	40.0	20.0	60.0	80.0
	MST	18.4	21.2	19.8	13.0
-3.5	% Dead	40.0	60.0	60.0	75.0
	MST	19.8	16.6	16.6	13.5
Exp. 2 HSV-2 i.v. infection					
-1.0	% Dead	100.0	ND	100.0	ND
	MST	7.5	ND	7.8	ND
-2.0	% Dead	100.0	ND	17.0	ND
	MST	10.7	ND	17.8	ND
-3.0	% Dead	17.0	ND	17.0	ND
	MST	18.0	ND	18.5	ND
-4.0	% Dead	0	ND	17.0	ND
	MST	>20.0	ND	18.5	ND
Exp. 3 HSV-2 i.v. infection					
-1.0	% Dead	100.0	100.0	ND	100.0
	MST	5.5	6.0	ND	4.0
-2.0	% Dead	100.0	100.0	ND	100.0
	MST	6.0	6.5	ND	5.0
-3.0	% Dead	100.0	100.0	ND	100.0
	MST	7.0	7.0	ND	6.5
-4.0	% Dead	33.0	100.0	ND	100.0
	MST	>21.0	10.0	ND	9.0
-5.0	% Dead	17.5	33.3	ND	83.3
	MST	>21.0	>21.0	ND	9.0

CD-1 mice were treated as indicated, infected i.p. or i.v. with HSV-2, mortality recorded and median survival calculated. Each group has 5-6 mice.

Table 8. Effect of i.v. Administration of DMDP Liposomes on Resistance to i.v. infection with Listeria monocytogenes (strain EGD) in CD-1 mice.

Group	Listeria CFU	% Dead	Median Survival Time
Saline	2.3×10^6	100.0	3.0
Free Liposomes	2.3×10^6	100.0	3.5
DMDP Liposomes	2.3×10^6	100.0	2.5
Saline	7.8×10^5	100.0	3.6
Free Liposomes	7.8×10^5	100.0	3.6
DMDP Liposomes	7.8×10^5	100.0	2.5*
Saline	2.3×10^5	83.3	6.0
Free Liposomes	2.3×10^5	100.0	4.5
DMDP Liposomes	2.3×10^5	100.0	2.5*
Saline	7.8×10^4	16.7	>14.0
Free Liposomes	7.8×10^4	16.7	>14.0
DMDP Liposomes	7.8×10^4	100.0*	2.5*
Saline	2.3×10^4	33.3	>14.0
Free Liposomes	2.3×10^4	33.3	>14.0
DMDP Liposomes	2.3×10^4	100.0*	2.5

CD-1 female mice, aged 6 weeks, were treated i.v. as indicated on days -3 and -1 before i.v.infection with the indicated dose of Listeria. Each group contained 6 mice.

*Statistically significant ($p<0.05$) as compared with saline and free liposome control groups.

4.0 Publications Relating to and Resulting from This Contract.

Volkman, A., N.C. Chang, P. Strausbauch and P.S. Morahan. Differential effect of chronic monocyte deprivation on macrophage populations. Lab. Invest. 49: 291-298, 1983.

Volkman, A., N-C. Chang, P. Strausbauch and P. Morahan. Maintenance of resident macrophage populations in monocyte depleted mice. In: Mononuclear Phagocyte Biology, ed. A. Volkman, Marcel Dekker, Inc., 1984.

Shibata, Y. and A. Volkman. The effect of bone marrow depletion on prostaglandin E producing suppressor macrophages in mouse spleen. J. Immunol. 135: 3897-3904, 1985.

Shibata Y. and A. Volkman. The effect of hemopoietic microenvironment on suppressor macrophages in the congenitally anemic mice of the genotype Sl/Sld. J. Immunol. 135: 3905-3910, 1985.

Shibata, Y., W.L. Dempsey, P.S. Morahan and A. Volkman. Selectively eliminated blood monocytes and splenic suppressor macrophages in mice depleted of bone marrow by strontium 89. J. Leuk. Biol. 38: 659-669, 1985.

Morahan, P.S., A. Volkman, W.L. Dempsey and J. Connor. Antimicrobial activity of various immunomodulators: independence from normal levels of circulating monocytes and NK cells. Infec. Immun. 51: 87-93, 1986.

Dempsey, W.L., A. Smith and P.S. Morahan. Effect of inapparent murine hepatitis virus infections on macrophages and host resistance. J. Leuk. Biol. 39: 559-566, 1986.

Morahan, P.S., E.R. Leake, D.J. Tenney and M. Sit. Comparative analysis of modulators of non-specific resistance against microbial infections. In: J. Majde, Ed., Immunological Adjuvants and Modulators of Non-Specific Resistance to Microbial Infections (Alan R. Liss, NY).

Shibata, Y., A.P. Bautista, S.N. Pennington, J.L. Humes and A. Volkman. Eicosanoid production by peritoneal and splenic macrophages in mice depleted of bone marrow by ^{89}Sr. Am. J. Pathol. 127: 75-82, 1987.

Morahan, P., A. Volkman, M. Melnicoff and W. Dempsey. Macrophage Heterogeneity, Chapter in: Macrophages and Cancer, eds. G.H. Heppner and A. Fulton, CRC Press Uniscience Series, Boca Raton, FL.

Shibata, Y. Restoration of prostaglandin-producing macrophages in

^{89}Sr-treated mice with bone marrow from Corynebacterium parvum primed donors. Submitted for publication in Journal of Immunology, 1987.

Shibata, Y. Humoral factors in the induction of prostaglandin E_2-producing macrophages in vitro. Submitted for publication in Journal of Immunology, 1987.

Shibata, Y. and A. Volkman. Restoration of prostaglandin releasing macrophage populations in lethally irradiated mice with spleen cells from bone marrow-depleted donors. Submitted for publication in the Journal of Immunology, 1988.

Shibata, Y. Effect of anti-class I MHC monoclonal antibody treatment on prostaglandin E_2 production by mononuclear phagocytes. Submitted for publication in the Journal of Immunology, 1988.

4.1 Presentations and Abstracts.

Connor, J., W.L. Dempsey, G.E. Ericsson, M. Ackermann, P. Hwu and P.S. Morahan. Effects of ^{89}Sr destruction of bone marrow on mononuclear phagocytes. Philadelphia Immunology Meeting, October 1983.

Morahan, P.S., J. Connor, G.E. Ericsson, W.L. Dempsey, A. Volkman and P. Hwu. Effects of ^{89}Sr destruction of bone marrow on mononuclear phagocytes. Fed. Proc., June 1984.

Morahan, P.S., W.L. Dempsey and A. Volkman. Use of ^{89}Sr to define the role of tissue macrophages, circulating monocytes and NK cells in natural and immunomodulator induced antiviral resistance. Symposium on Pathobiology and Immunopathology of Virus Infections, Sendai, Japan, September 1984.

Morahan, P.S. and A. Volkman. Effects of bone marrow destruction with ^{89}Sr on natural resistance to viruses and resistance induced by immunomodulators. Sixth International Congress of Virology, Sendai, Japan, September 1984.

Ackermann, M.F., W.L. Dempsey, P. Hwu, D. Tenney, E. Leake and P.S. Morahan. Effect of PMN depletion on MØ activation by P. acnes. Eastern Pennsylvania Branch of the American Society of Microbiology, February 1985. (Appendix 5)

Tenney, D.J., M.F. Sit, E.R. Leake, M.T. Largen and P.S. Morahan. Effect of ^{89}Sr on protein synthesis and intrinsic resistance of peritoneal macrophages to HSV-1. Reticuloendothelial Society annual meeting, July 1985. (Appendix 6)

Ackermann, M.F., W.L. Dempsey, P. Hwu, D. Tenney, E. Leake and P.S. Morahan. Effect of PMN depletion on MØ activation by P. acnes.

Eastern Pennsylvania Branch of the American Society of Microbiology, February 1985.

Tenney, D.J., M.F. Sit, E.R. Leake, M.T. Largen and P.S. Morahan. Effect of ^{89}Sr on protein synthesis and intrinsic resistance of peritoneal macrophages to HSV-1. Reticuloendothelial Society annual meeting, August 1985.

Shibata, Y., A. Bautista, S. Pennington, J. Humes and A. Volkman. Effect of bone marrow depletion with ^{89}Sr on eicosanoid production by murine peritoneal and splenic macrophages. International Congress of Immunology, July 1986.

Volkman, A., Y. Shibata, W. Dempsey and P. Morahan. Effects of bone marrow ablation on compartmental prostaglandin synthesis by mononuclear phagocytes. American Society for Microbiology symposium, November 1986.

Shibata, Y. and A. Volkman. Restoration of macrophage populations in mouse radiation chimeras established with spleen cells from bone marrow-depleted donors. Eleventh International RES Congress, October 1987.

5.0 Legends for Text Figures.

Figure 1. Nuclear magnetic resonance protein spectrum of liposome preparation containing ricin. 400 MHz NMR frequency (see text).

Figure 2. Liposomes (PC/PA/cholesterol) were prepared with ^{3}H-cholesterol as a tracer for in vitro uptake by three different types of mononuclear phagocytes, resident peritoneal MØ (RPM), thioglycollate elicited peritoneal MØ (EXM) and the P388D1 MØ-like cell line. Aliquots of the liposome suspension were filtered through Nucleopore polycarbonate membranes with porosities of 0.6μ and 0.2μ. Background counts of unlabeled liposomes did not exceed 17 CPM. The RPM and P388D1 cells appear to behave in a like manner with a mild reduction in the uptake of liposomes following removal of the largest multilamellar liposomes; the EXM appear unaffected by the procedure.

Figure 3. The graph compares the incorporation of ^{3}H-leucine, 1μCi/ml, by plastic-adherent MØ of different types following an 18h incubation with and without liposomes. RPM, resident peritoneal MØ; EXM, thioglycollate-elicited (4 day) peritoneal exudate MØ; P388D1, MØ-like cells of the P388D1 line. Results of 2 replicates for each class of MØ are shown. In this example, multilamellar liposomes were prepared with "pure" PC and dialyzed before being added to the incubation medium. No suppressive effect on protein synthesis is shown.

Figure 4. The contrasts between protein synthesis by MØ shown in Fig. 3 are examined at incremental intervals of incubation from 1 to 24 hrs in ^{3}H-leucine, 0.1μCi/culture dish. Data for peritoneal MØ are shown in the upper box (4A) and for P388D1 cells in the lower box. The levels of ^{3}H-leu incorporation stand approximately in the same ratios as shown in Fig. 3. No liposomes were employed in this experiment.

Figure 5. Percent toxicity, measured by the 4 hr release of ^{51}Cr (see text), is plotted against increasing concentrations of amphotericin B. Resident peritoneal MØ were plated in 35mm plastic dishes at 1 x 10^{6}/ml to the point of adherence before the addition of Amphotericin B to the culture medium.

Figure 6. Data in Fig. 6a show the toxic effect of increasing concentrations of whole ricin on adherent P388D1 cells incubated for varying intervals in plastic dishes. Toxicity is measured by a one hour ^{3}H-leu uptake compared with the ricin-free control. A ricin concentration of 10μg/ml is approximately 1.5 x 10^{-7} molar. A near maximum effect is seen at the lowest dose employed corresponding to approximately 4 x 10^{-9} molar ricin. Fig. 6b shows corresponding data for ^{3}H-leu uptake by RPM in a 24 hour incubation.

Figure 7. Data compare the relative toxicity of whole ricin and ricin A-chain (RAC) in vitro for resident and exudate types of mouse peritoneal MØ (7a) and for P388D1 cells (7b). Culture conditions

are as before. Uptake of ^{3}H-leu, 0.1μCi/ml of culture medium is used to monitor impaired protein synthesis. Note the more marked toxicity of "free" RAC.

Figure 8. The toxic effects of ricin and ricin A-chain on peritoneal MØ in vivo are compared by counting the residual MØ in lavage specimens approximately 18h after ip injection of these agents. Means ± SD of counts
corresponding to log incremental doses are shown. Compare data with Fig. 7a.

Figure 9. Data show the effects of 0.4ml of incremental doses of free ricin given IP. Peritoneal lavage was performed one day later. Means ± SD of mice/data point are shown in total nucleated cell counts and counts of polymorphonuclear leukocytes (PMN), macrophages (MAC) and lymphocytes (Ly). The increase in PMN reflects the highly inflammatory nature of ricin; whereas the almost linear decline in MØ numbers probably reflects toxicity due to specific binding of ricin. See text for additional discussion.

Figure 10. Data show the effects of liposome-encapsulated ricin and ricin A-chain on nucleated mouse peritoneal cell populations 24 hrs after IP administration of 0.4 ml of each agent. Total cells; PMN, polymorphonuclear leukocytes; MAC, macrophages; Ly, lymphocytes. Labeling along the abscissa indicates contents of liposomes.

Figure 11. Data show means ± SD for peritoneal cell counts 24 hrs following the ip instillation of DMDP-liposomes. See text for details.

Figure 12, a-e. The effects of ip liposome-encapsulated ricin, ricin A-chain (RAC) and DMDP were compared in mice treated on day 0 with ^{88}Sr or ^{89}Sr and day 9 with ip toxins/liposomes. Controls included panels of mice injected ip with sterile saline, empty liposomes and with free ricin. Levels of blood monocytes and peritoneal lavage MØ are shown on the day of sacrifice as indicated on the abscissa.

Figures 12a and 12b show the effects on blood monocytes: Figures 12c-e show effects on peritoneal MØ. All data are expressed as mean percent change with reference to ^{88}Sr-saline. Figure 12a shows profound monocyte depletion in the ^{89}Sr-saline group with slowly progressive recovery. Monocyte depression was mildly depressed on days 10/11 and 27/28 but these may be fluctuations about the normal. The same may be said for ^{88}Sr-free ricin but these mice died within 72 hours of receiving the toxin and are difficult to evaluate. The ^{89}Sr mice which received liposomes or free ricin maintained low blood monocyte levels. Figure 12b shows again that monocyte counts in all ^{89}Sr treated mice are low but some of the ^{88}Sr groups show elevations in the counts.

Figure 12c shows changes in numbers of peritoneal MØ corresponding to the treatments and intervals shown in 12a and 12b. The toxic effect of ricin is seen in the ^{88}Sr and ^{89}Sr mice. The effects of empty liposomes are milder and less consistent.

In Figure 12d, the most pronounced MØ depression is seen in ^{89}Sr treated mice with apparent elicitation at some intervals in the ^{88}Sr groups.

Figure 12e shows that DMDP-liposomes resulted in apparent mild elicitation in ^{88}Sr and ^{89}Sr groups except for the 13/14 day ^{89}Sr and the 24/25 day ^{88}Sr.

Figure 13a-d. Data show survival of ^{89}Sr treated mice and their ^{88}Sr controls all of which later received liposome encapsulated ricin or empty liposomes ip followed by varying dilutions of ip Listeria monocytogenes, 24 hrs still later. The approximate LD_{50} is represented by the 10^{-3} dilution. Resistance to infection with Listeria monocytogenes appears to be more closely related to treatment with ricin-liposomes than to treatment with ^{89}Sr although Figures 13a and 13b suggest slightly better survival among ^{88}Sr controls than among the ^{89}Sr mice.

Figures 14a-c. The data in this series show percent survival versus time after the IP administration of varying dilutions of L. monocytogenes from 10^{-2} to 10^{-7} in CD-1 mice. Listeria was given 13 days after IV ^{88}Sr or ^{89}Sr; liposome encapsulated ricin or empty liposomes were given IP 24 hrs after Listeria. The nominal LD_{50} was 10^{-4}; survival of ^{88}Sr and ^{89}Sr mice, however, was not greatly affected by infection (data for ^{88}Sr-empty liposomes not shown). Figures 13B and 13C show that the principal determinant of altered resistance was the administration of ricin liposomes and not Listeria.

Figure 15a-b. In 15a, the data for the ^{89}Sr control do not show blood monocyte depletion as is also the case in the empty liposome controls. Most of the depressions in the blood monocyte counts are associated with ricin-liposome administration. It is interesting, however, that the elevation in the blood monocyte count, often seen in Listeria infection, appears only in the ^{89}Sr-ricin-liposome group.

Figure 15b shows a significant MØ-depressing effect of ricin-liposomes only in the panels of mice not treated with Listeria. Contrary to our usual observations, ip Listeria did not elicit a MØ influx in the ^{88}Sr-mice.

Figure 1

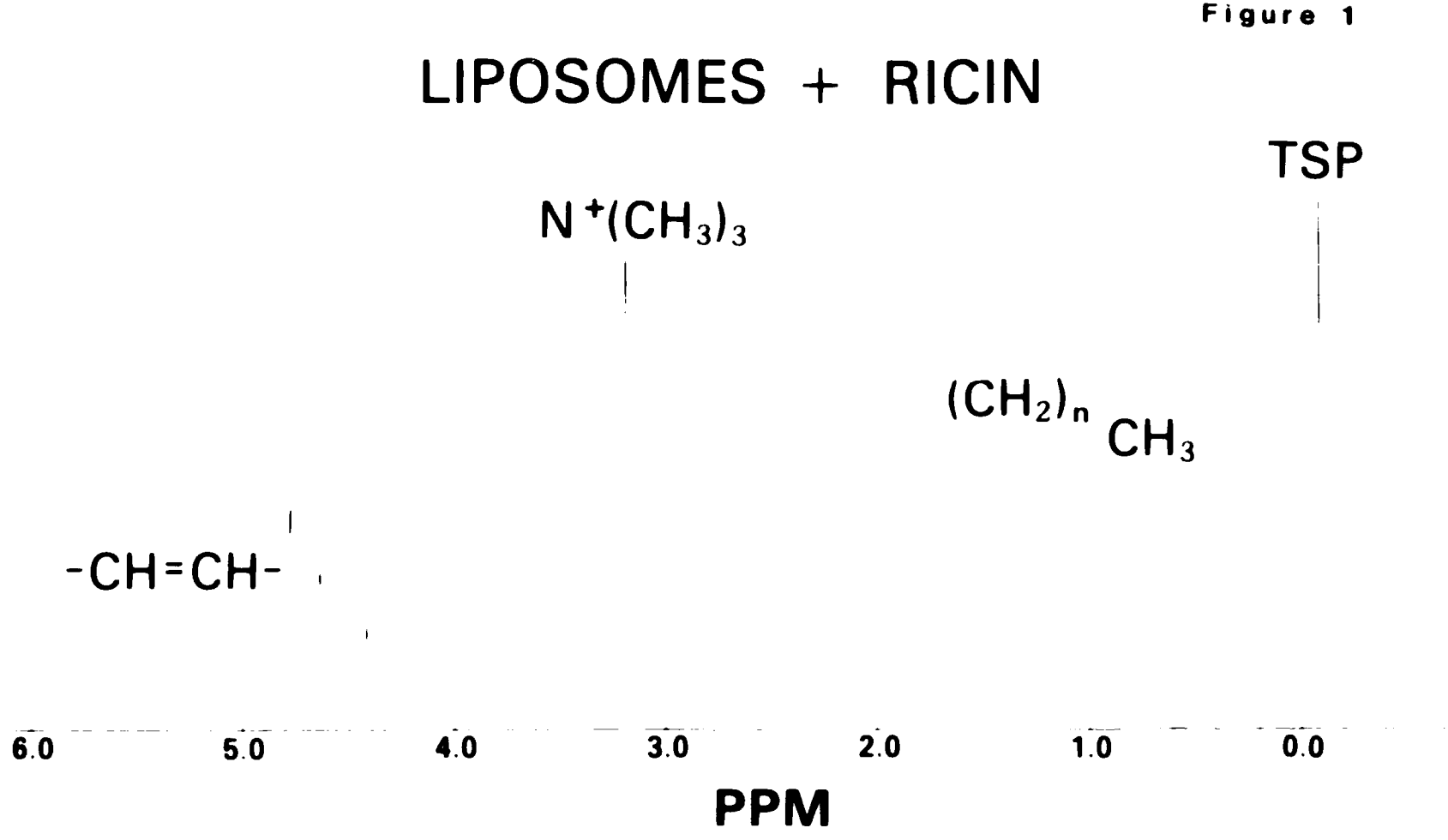

LIPOSOMES + RICIN
TSP
N+(CH3)3
(CH2)n CH3
-CH=CH-
6.0
5.0
4.0
3.0
2.0
1.0
0.0
PPM

Figure 2

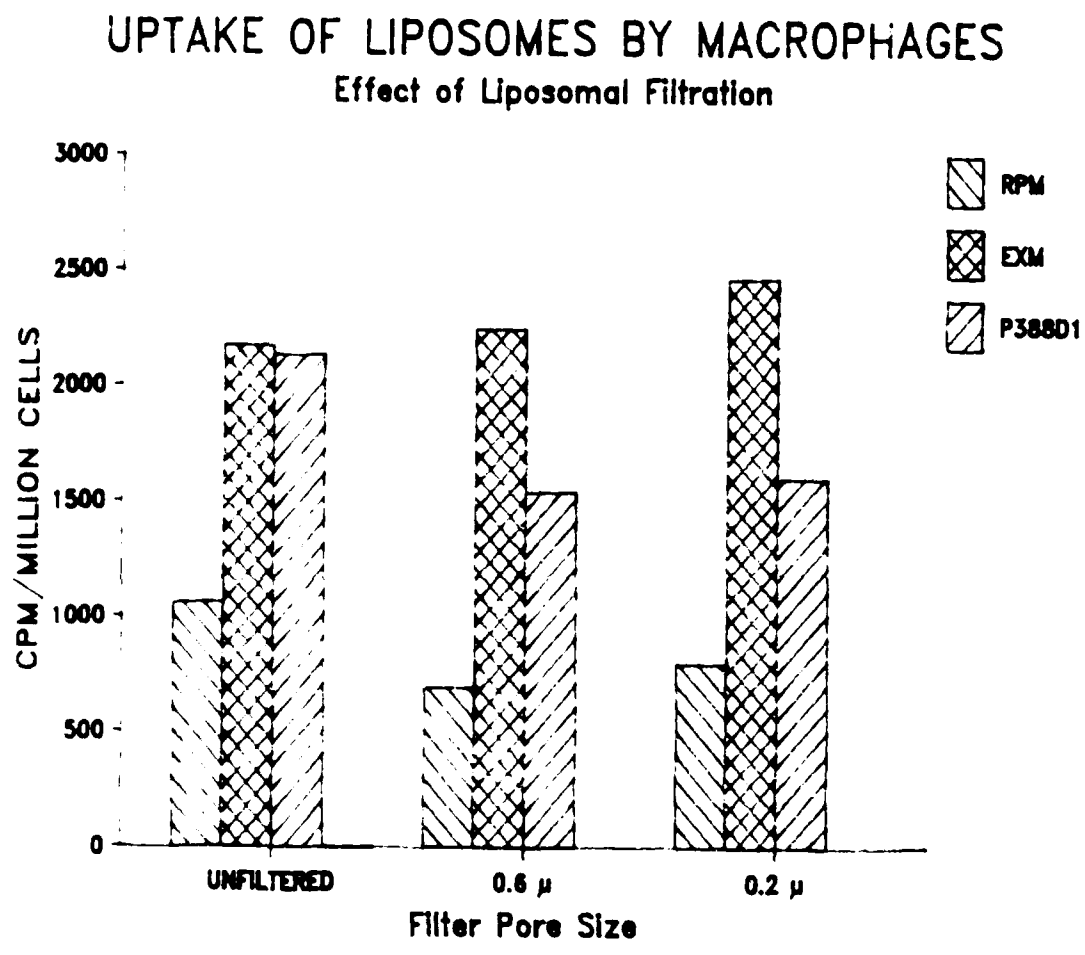

UPTAKE OF LIPOSOMES BY MACROPHAGES
Effect of Liposomal Filtration
CPM/MILLION CELLS
3000
2500
2000
1500
1000
500
0
RPM
EXM
P388D1
UNFILTERED
0.6 μ
0.2 μ
Filter Pore Size

Figure 3

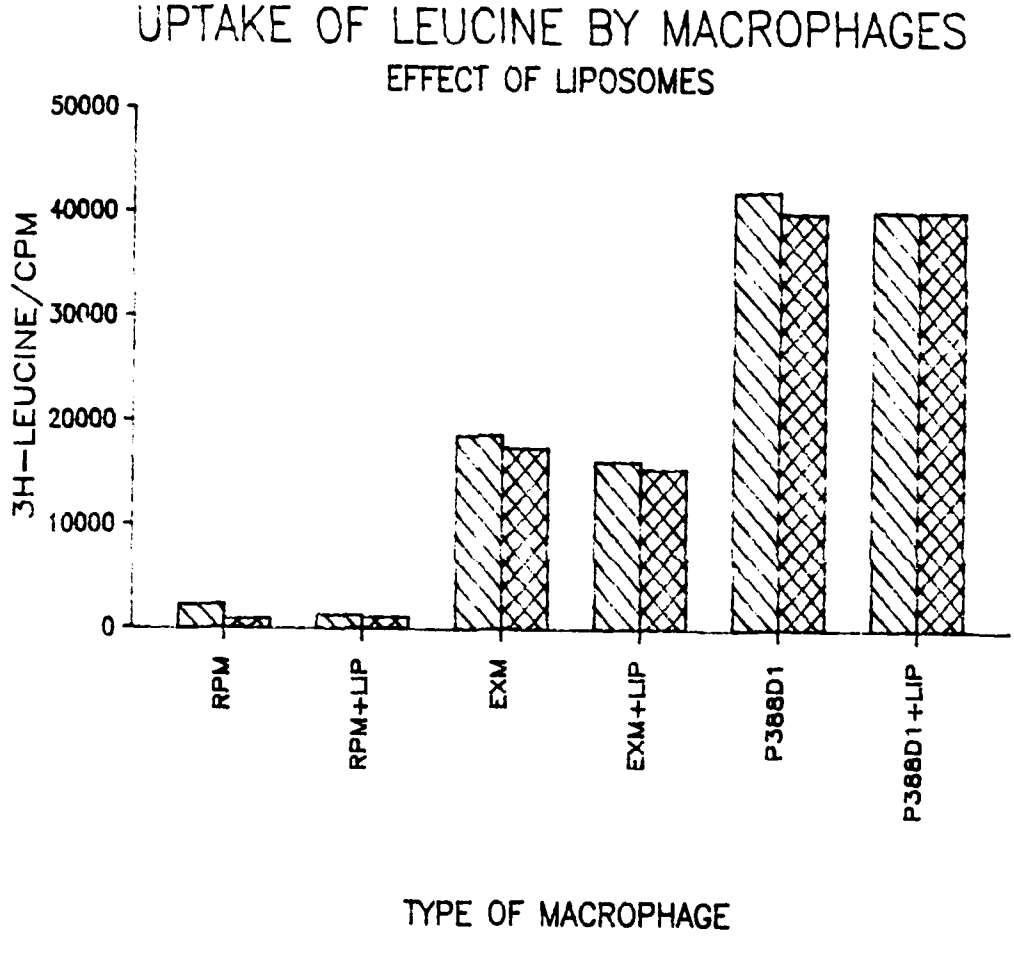

UPTAKE OF LEUCINE BY MACROPHAGES
EFFECT OF LIPOSOMES
3H-LEUCINE/CPM
50000
40000
30000
20000
10000
0
RPM
RPM+LIP
EXM
EXM+LIP
P388D1
P388D1+LIP
TYPE OF MACROPHAGE

Figure 4a

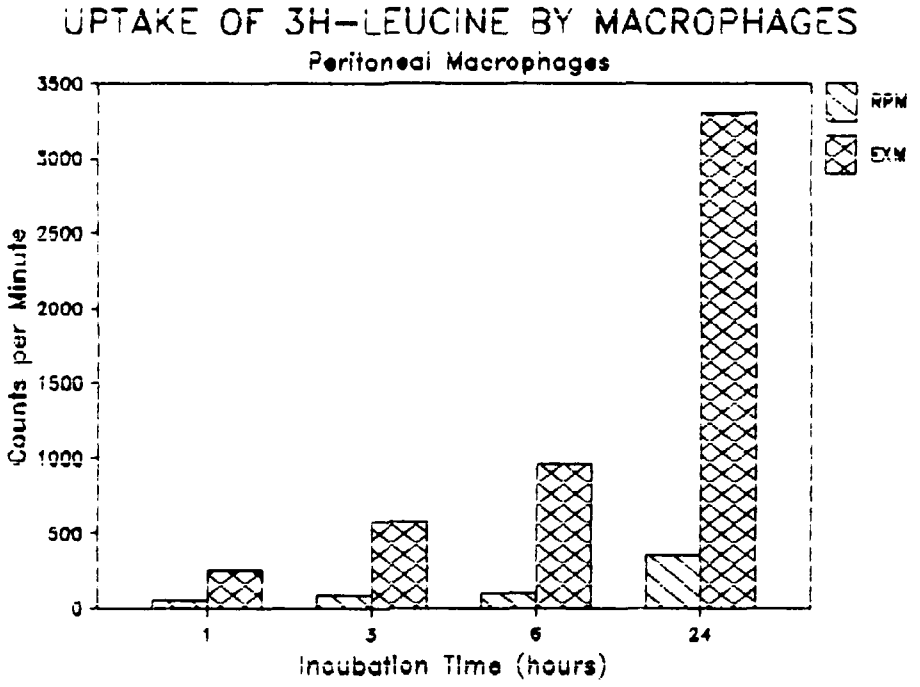


Figure 4b

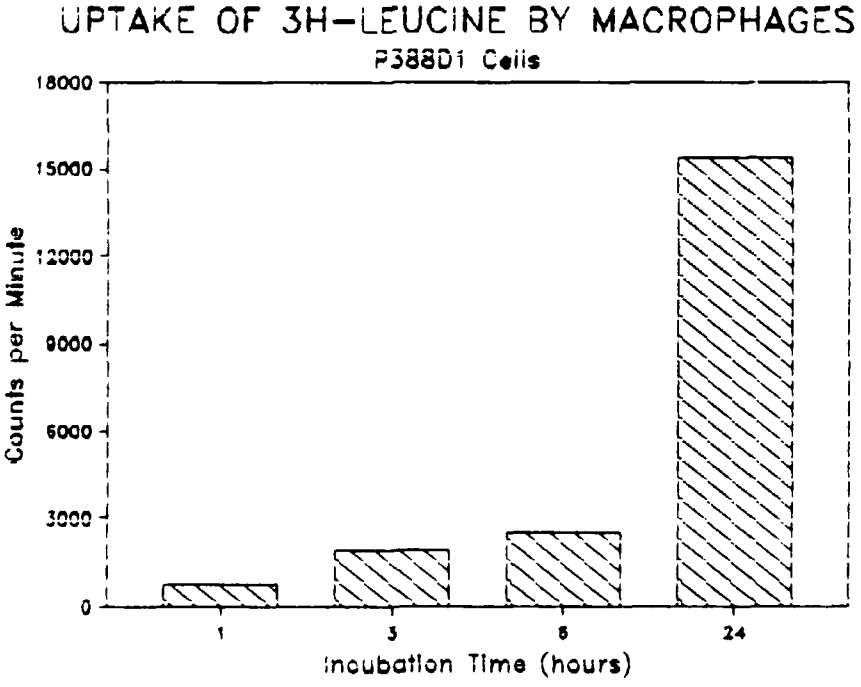


Figure 5

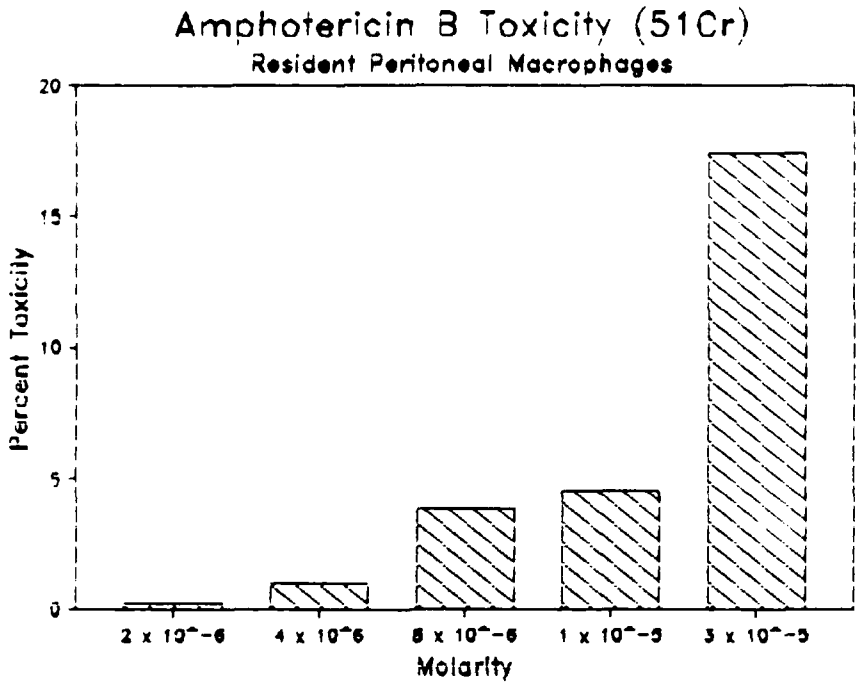

Amphotericin B Toxicity (51Cr)
Resident Peritoneal Macrophages
Percent Toxicity
20
15
10
5
0
2 x 10^-6
4 x 10^6
8 x 10^-6
1 x 10^-5
3 x 10^-5
Molarity

Figure 6a

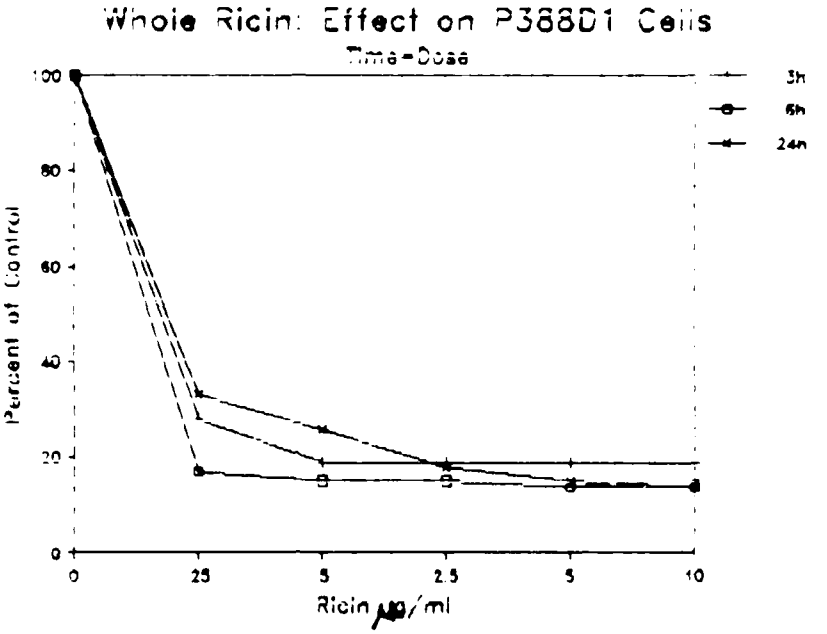


Figure 6b

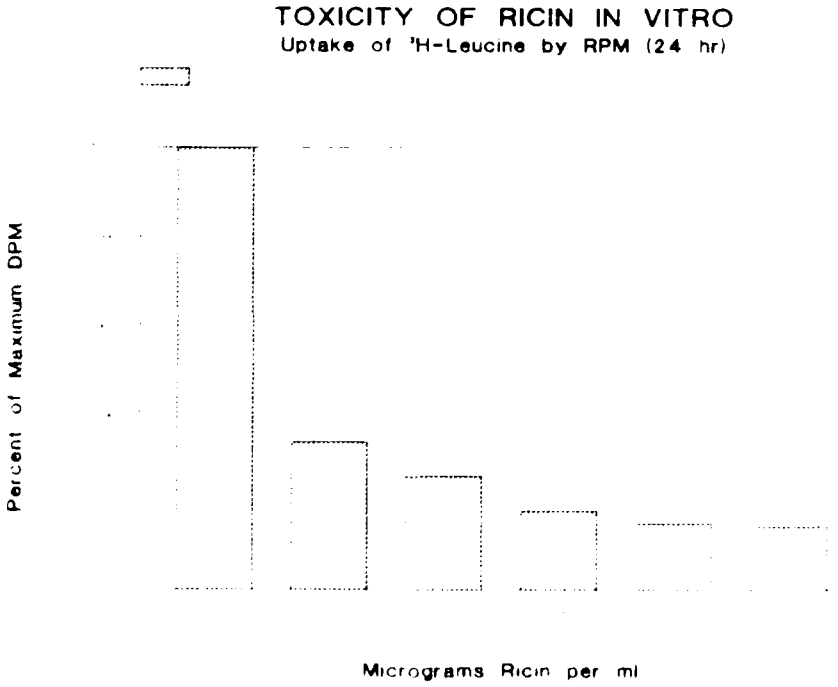


Figure 7a

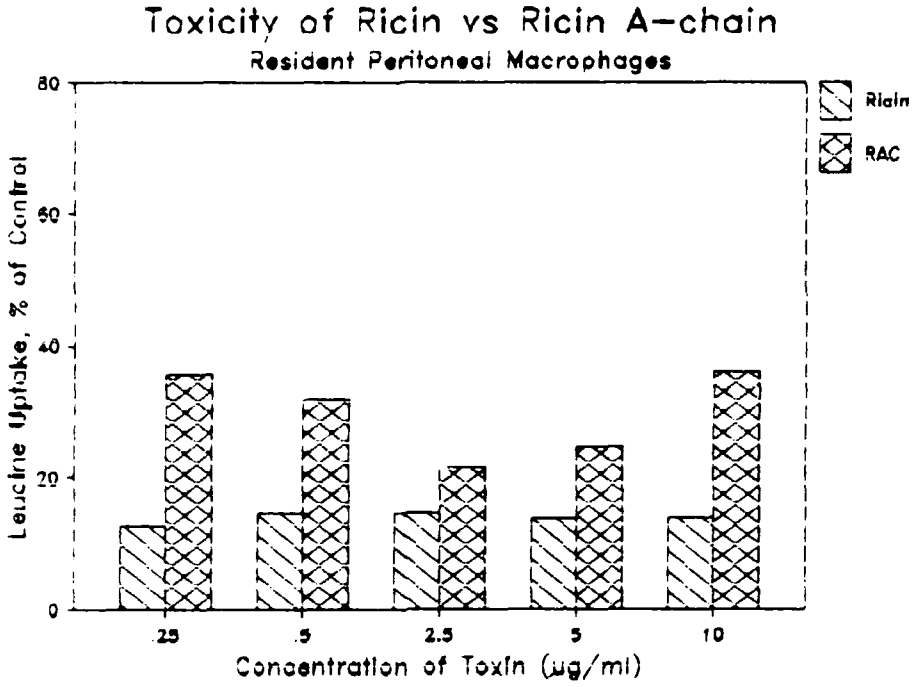


Figure 7b

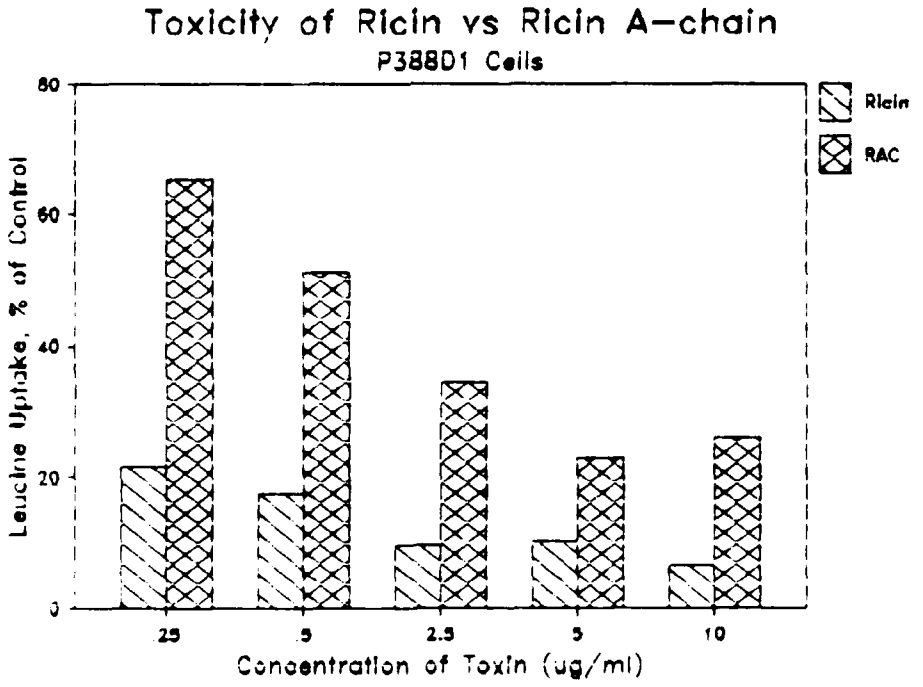


Figure 8

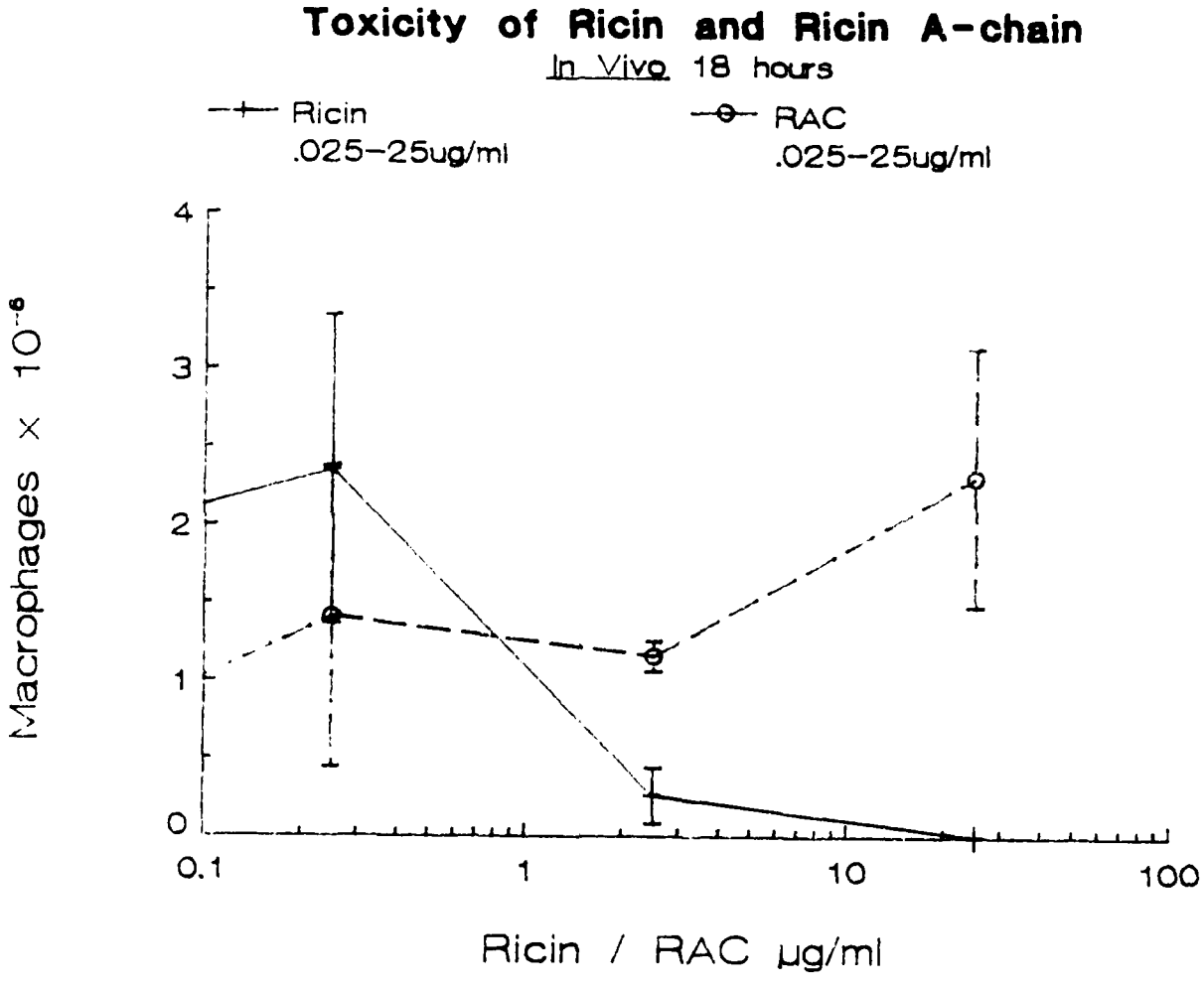

Toxicity of Ricin and Ricin A-chain
In Vivo 18 hours
Ricin
.025–25ug/ml
RAC
.025–25ug/ml
Macrophages x 10⁻⁶
4
3
2
1
0
0.1
1
10
100
Ricin / RAC µg/ml

Figure 9

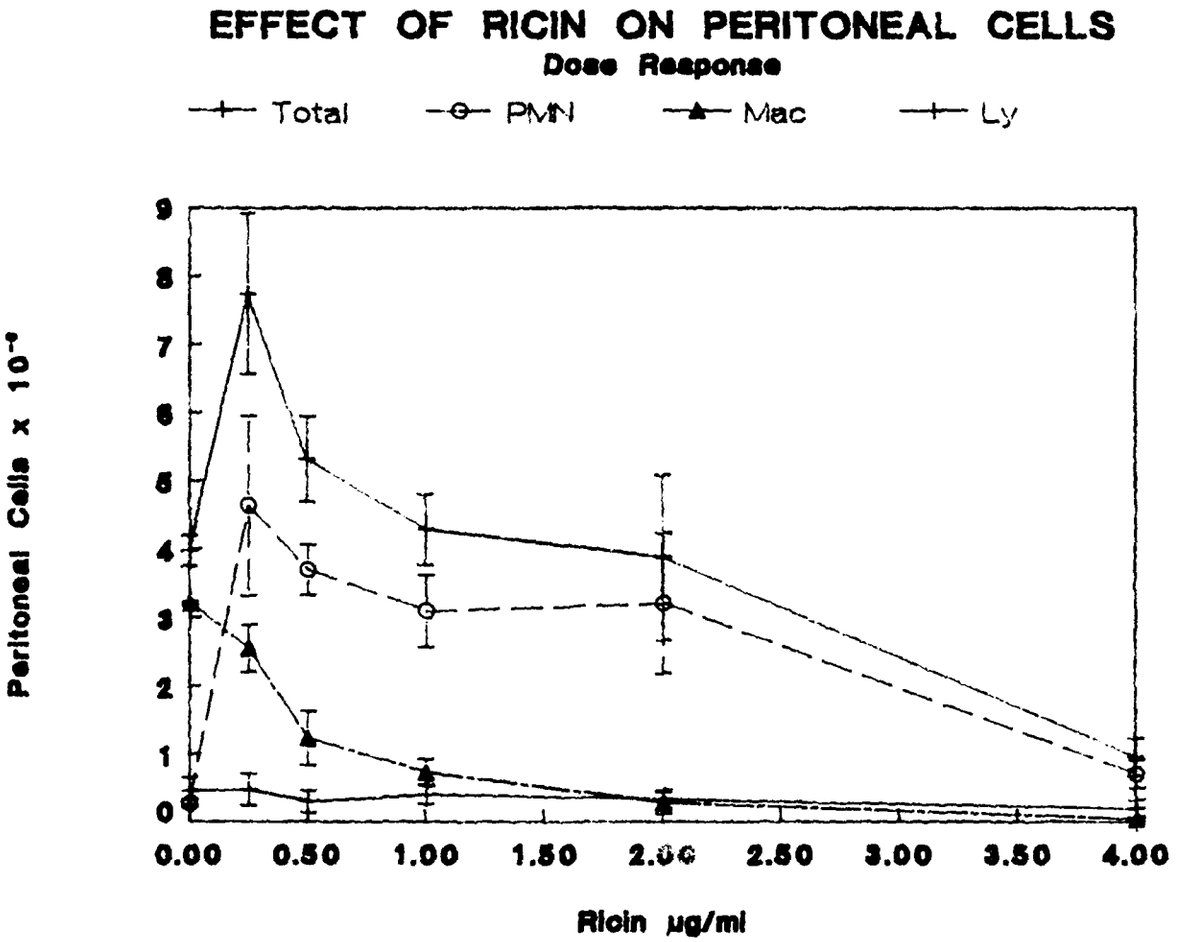

EFFECT OF RICIN ON PERITONEAL CELLS
Dose Response
Total
PMN
Mac
Ly
Peritoneal Cells x 10⁻⁶
9
8
7
6
5
4
3
2
1
0
0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
Ricin µg/ml

Figure 10

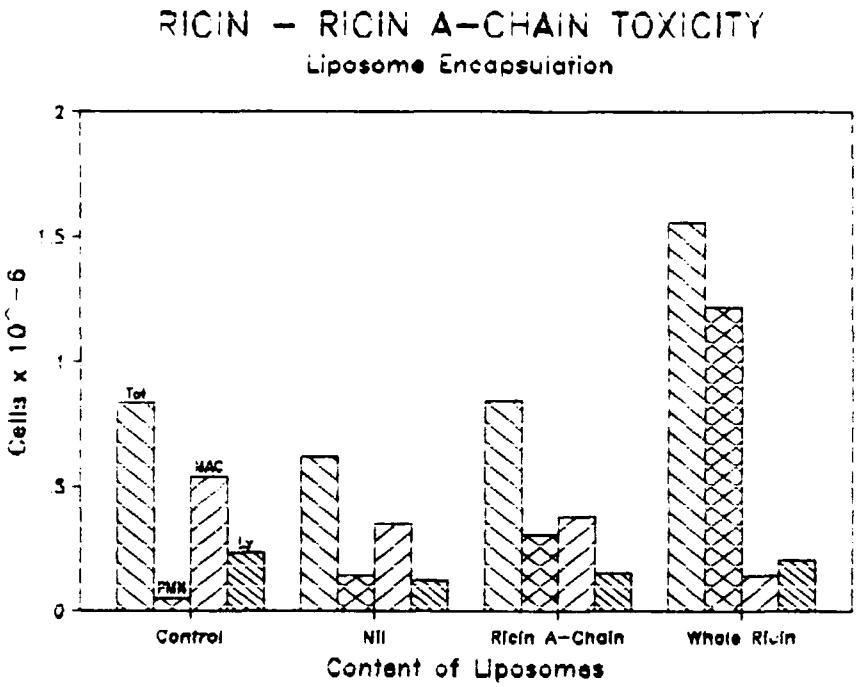

RICIN – RICIN A–CHAIN TOXICITY
Liposome Encapsulation
Cells x 10^-6
2
1.5
1
.5
0
Tot
MAC
PMN
Control
Nil
Ricin A–Chain
Whole Ricin
Content of Liposomes

Figure 11

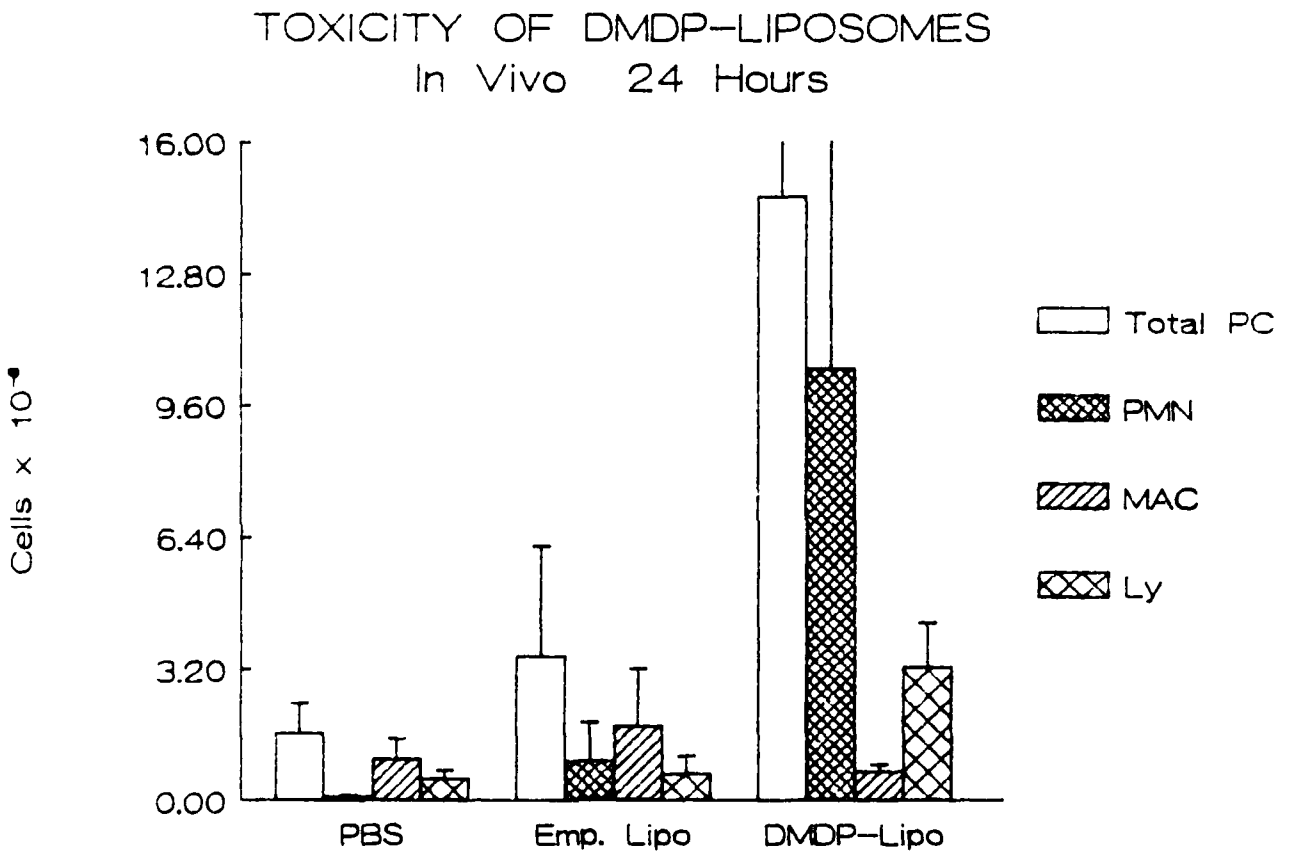

TOXICITY OF DMDP-LIPOSOMES
In Vivo 24 Hours
Cells x 10⁻⁴
16.00
12.80
9.60
6.40
3.20
0.00
PBS
Emp. Lipo
DMDP-Lipo
Total PC
PMN
MAC
Ly

Figure 12a

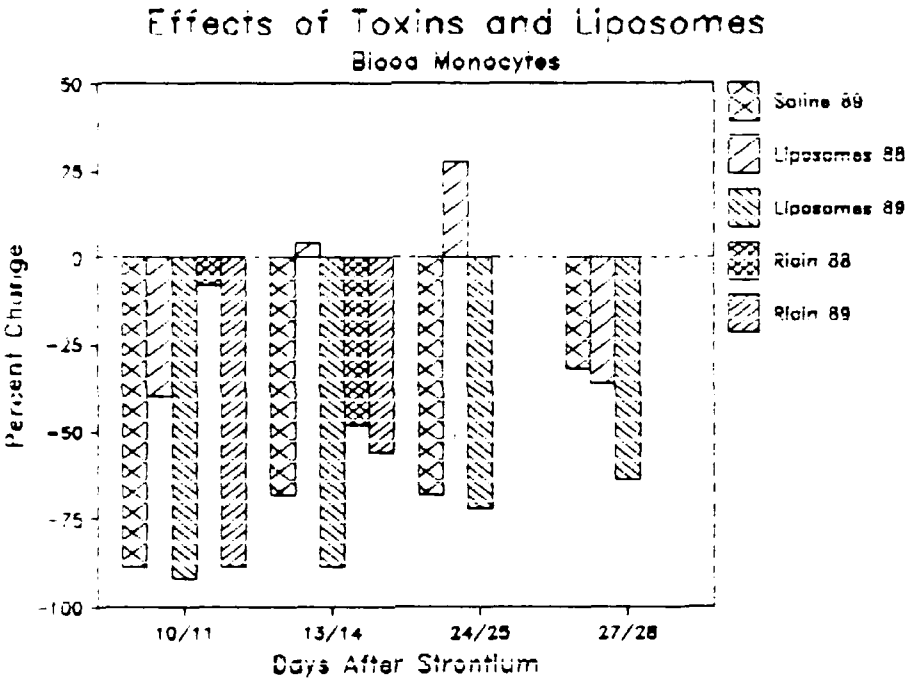


Figure 12b

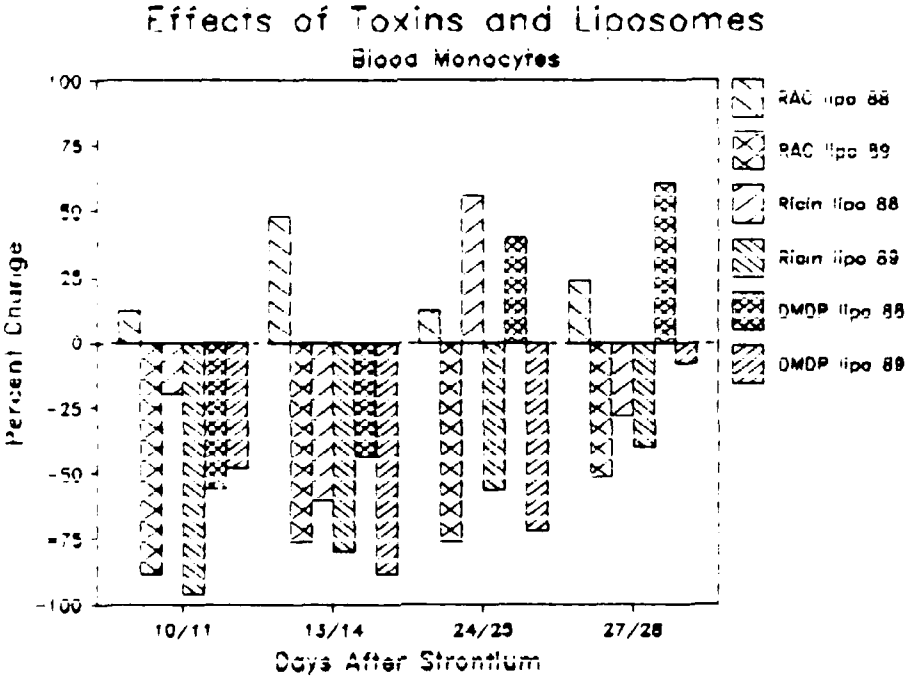


Figure 12c

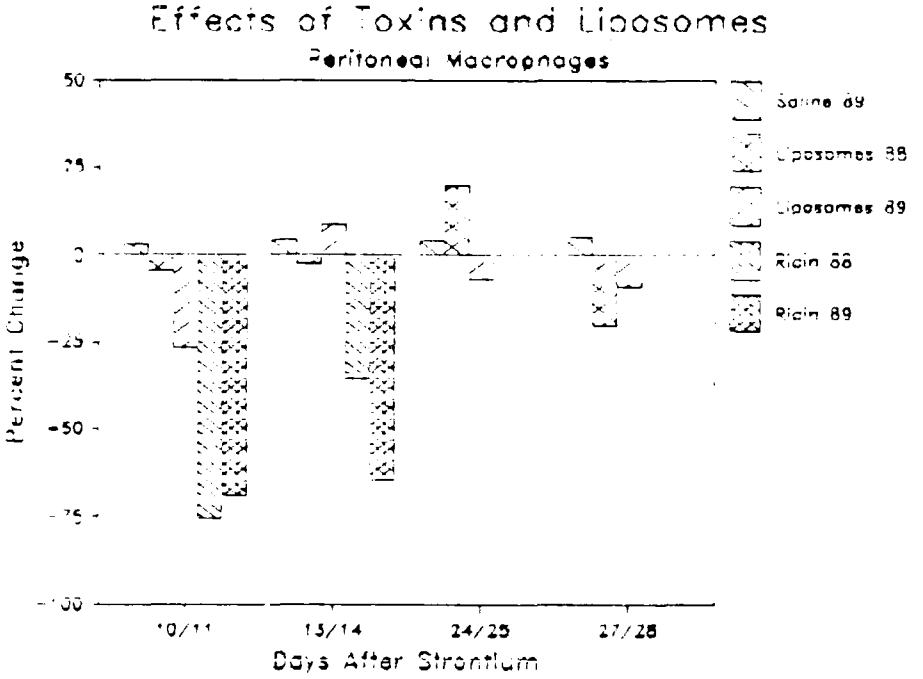


Figure 12d

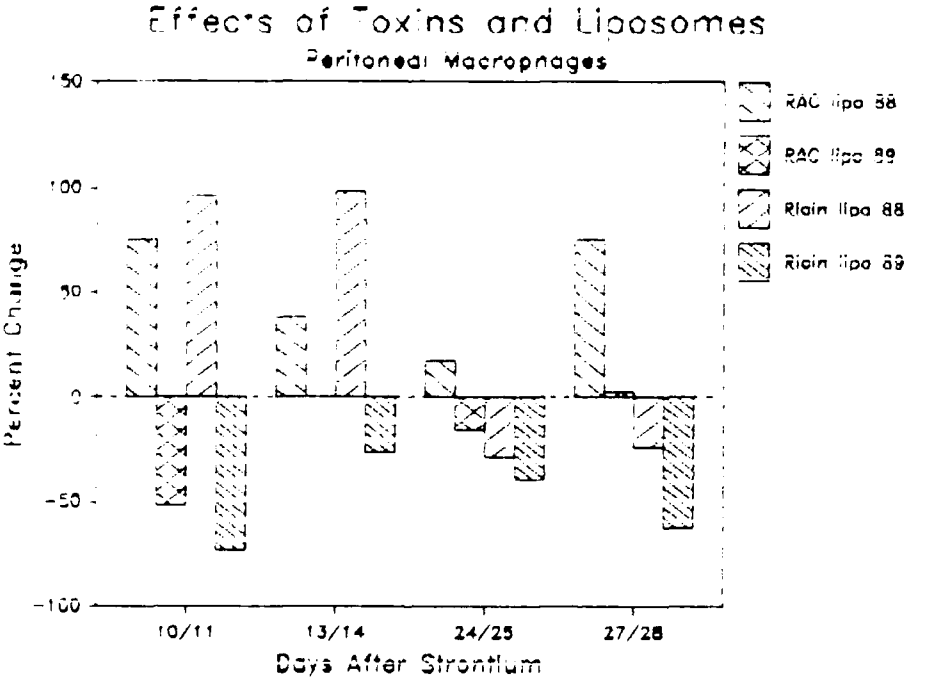


Figure 12e

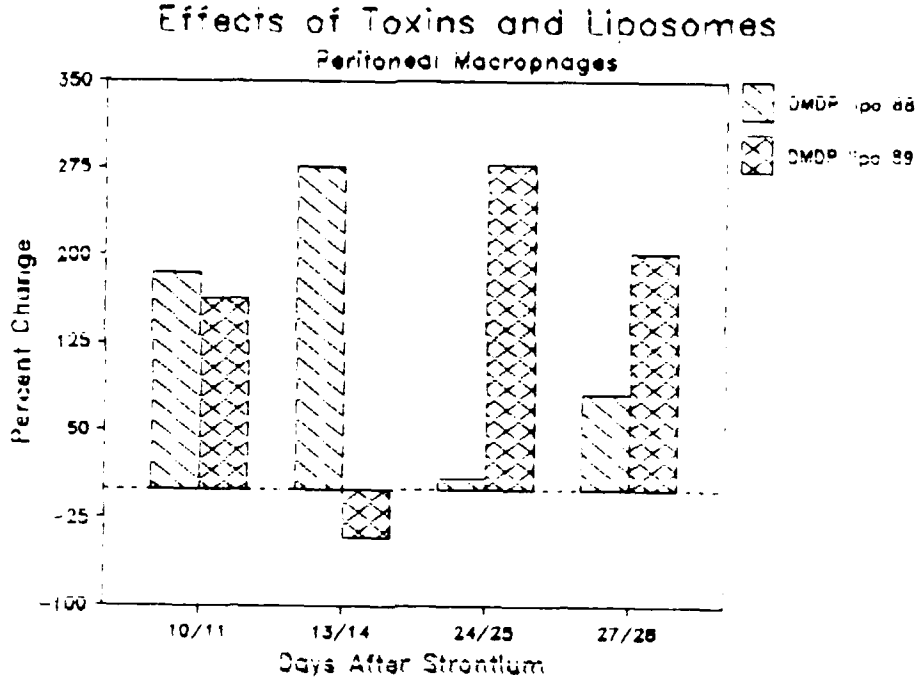

Effects of Toxins and Liposomes
Peritoneal Macropnages
350
275
200
125
50
-25
-100
Percent Change
10/11
13/14
24/25
27/28
Days After Strontium
DMDP lipo 88
DMDP lipo 89

Figure 13a

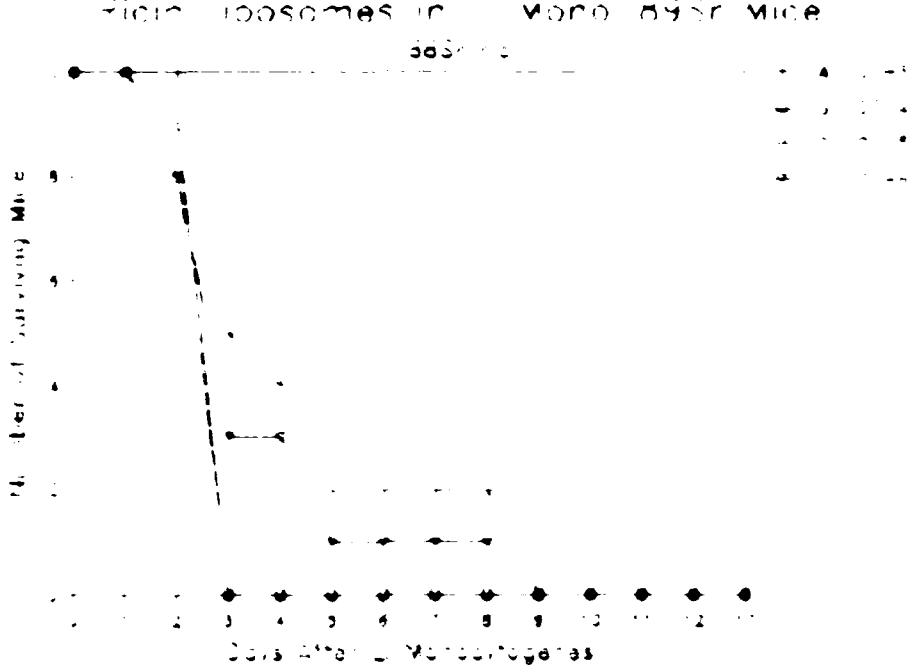


Figure 13b

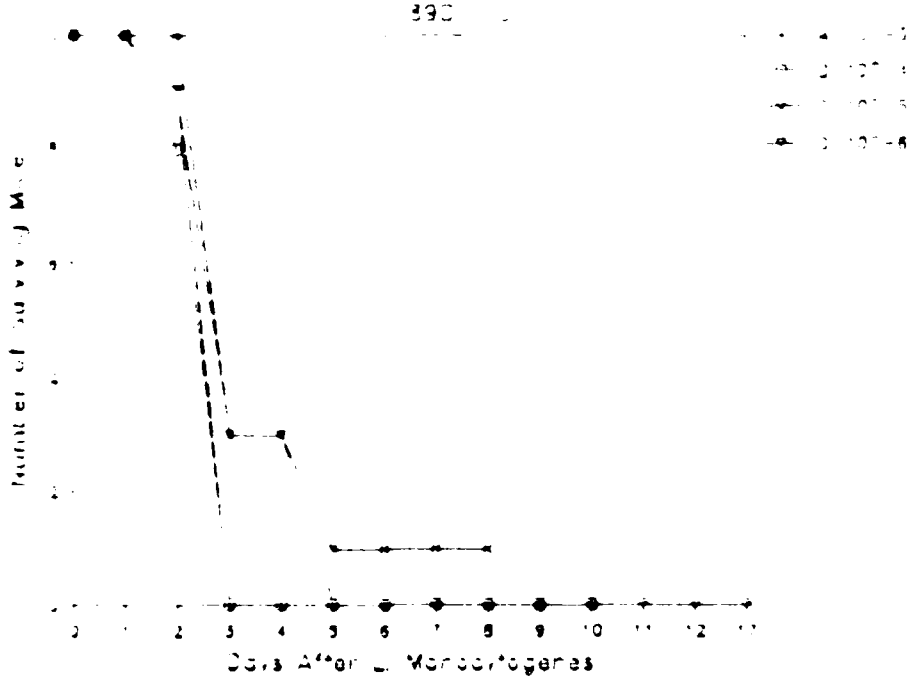


Figure 13c

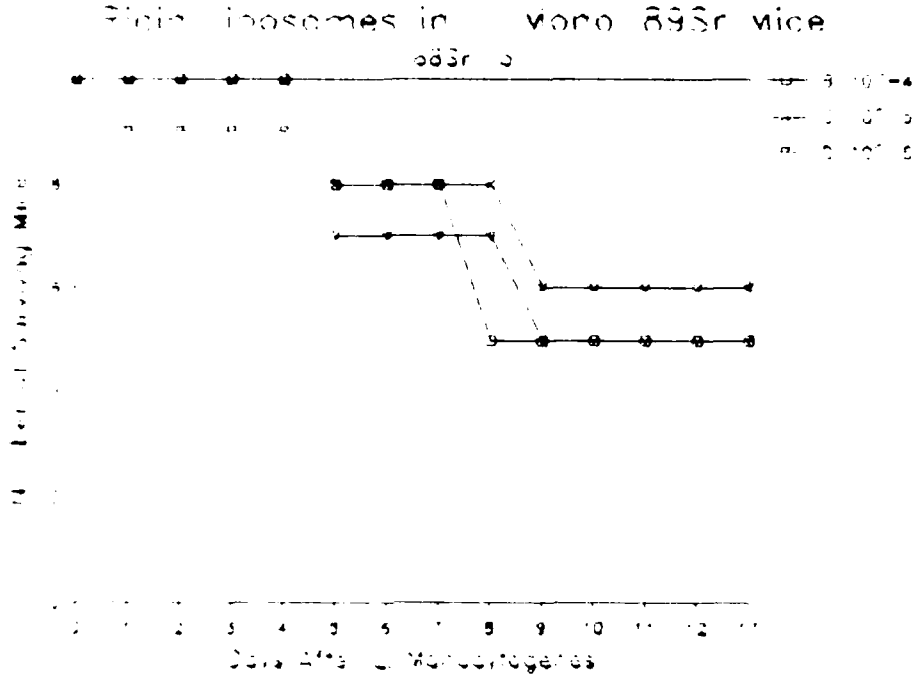


Figure 13d

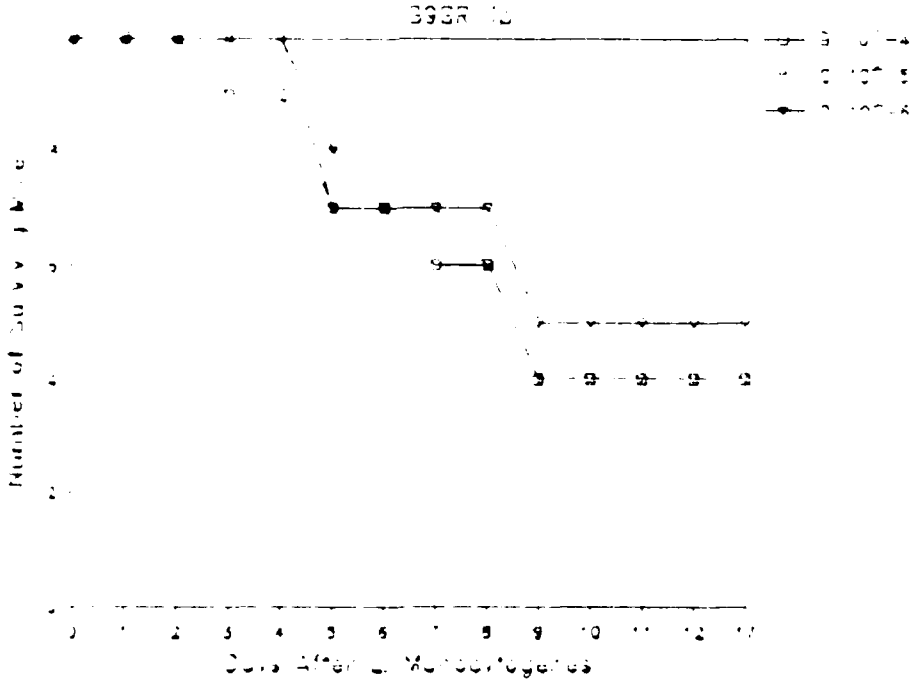


Figure 14a

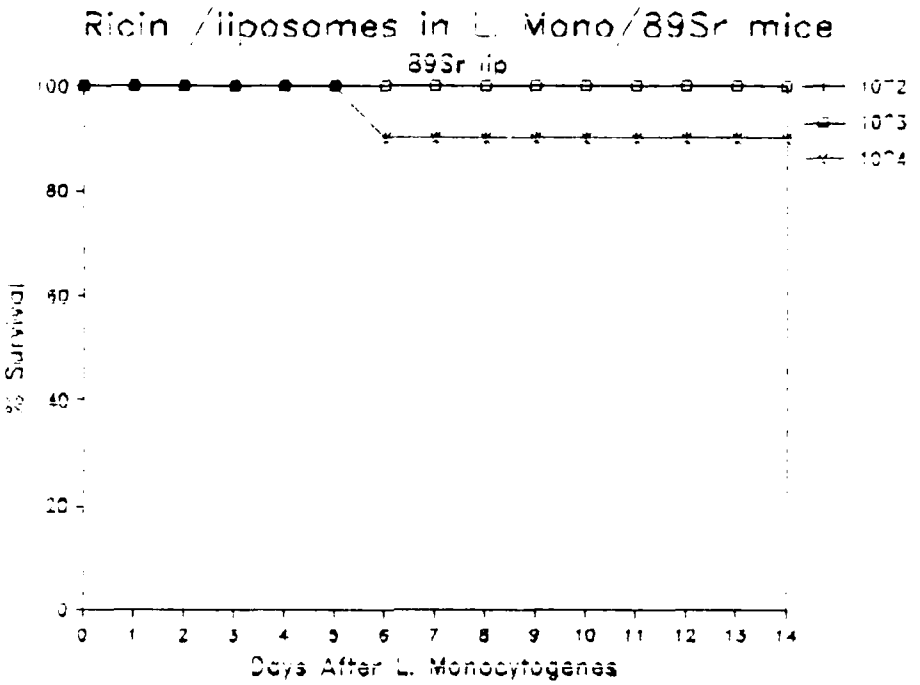

Ricin /liposomes in L. Mono/89Sr mice
89Sr ip
100
80
60
40
20
0
% Survival
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Days After L. Monocytogenes

Figure 14b

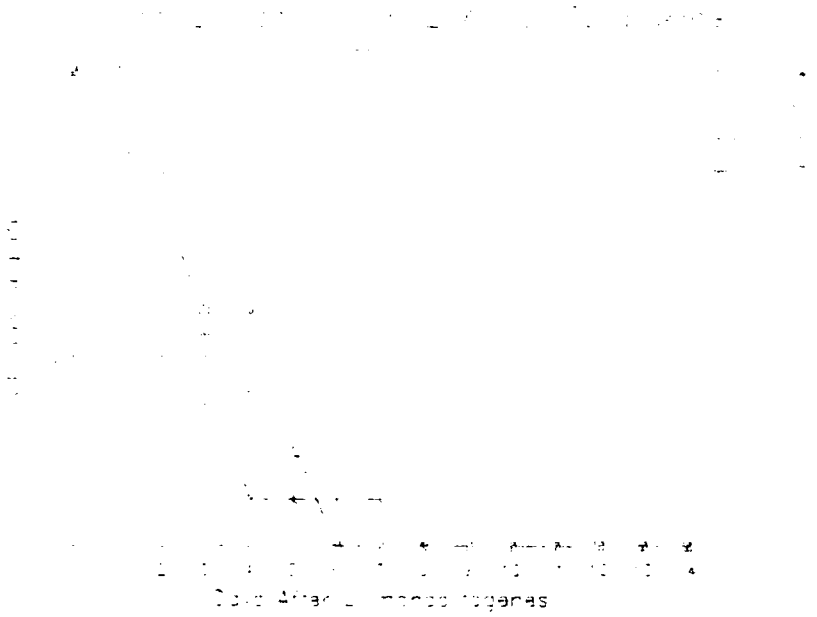


Figure 14c

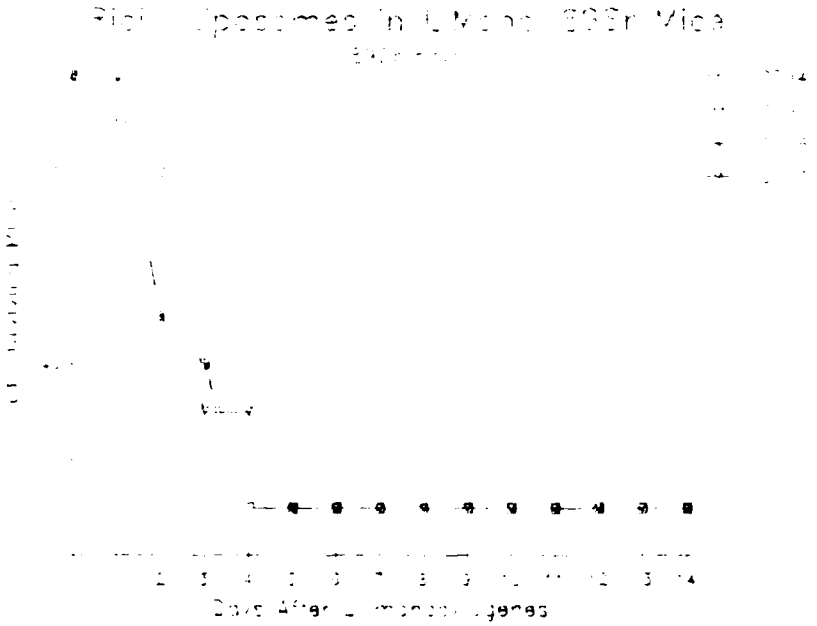


Figure 15a

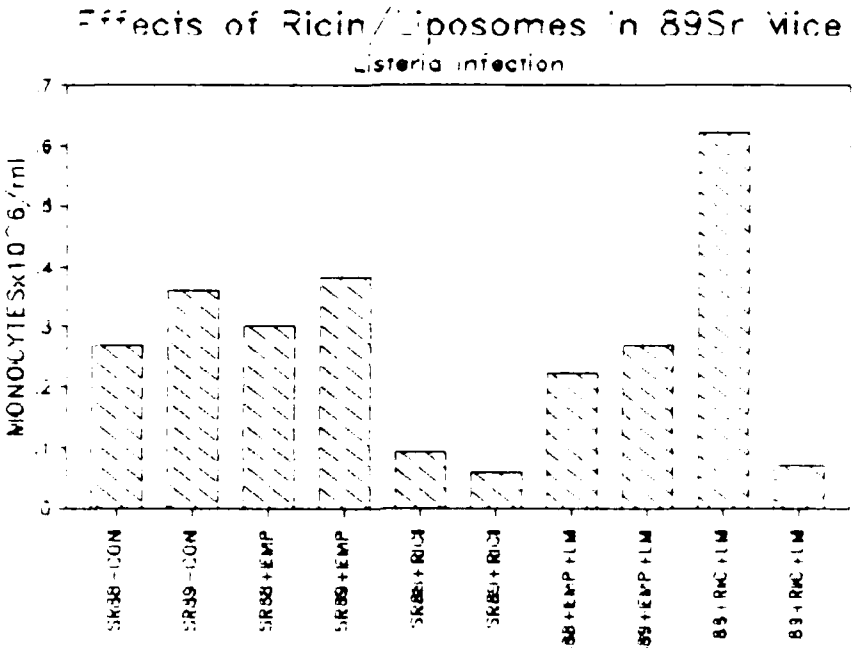
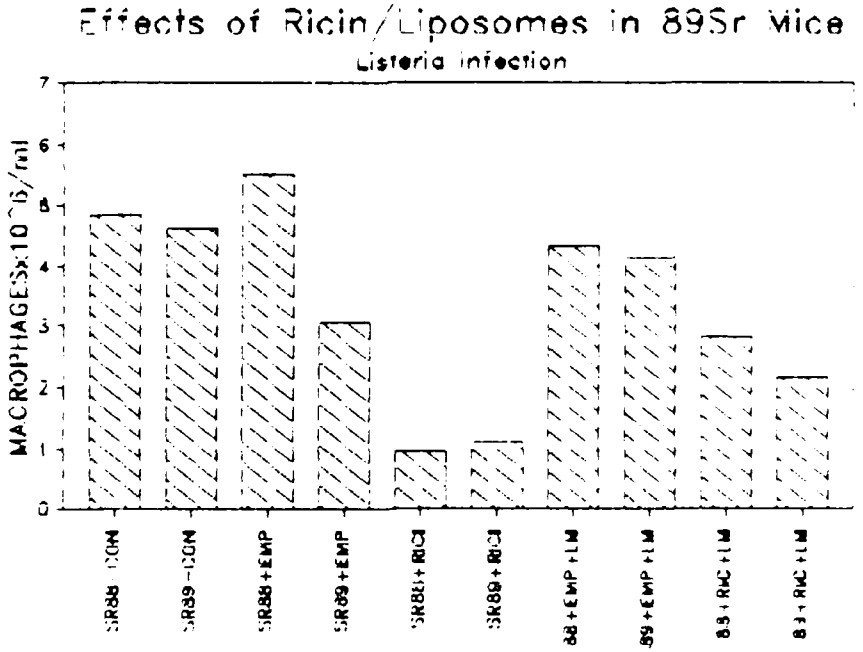


Figure 15b



(U)

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a REPORT SECURITY CLASSIFICATION (U)	1b RESTRICTIVE MARKINGS NA
2a SECURITY CLASSIFICATION AUTHORITY NA	3 DISTRIBUTION/AVAILABILITY OF REPORT Distribution Unlimited
2b DECLASSIFICATION/DOWNGRADING SCHEDULE NA	
4 PERFORMING ORGANIZATION REPORT NUMBER(S) Medical College of Pennsylvania	5 MONITORING ORGANIZATION REPORT NUMBER(S) NA
6a NAME OF PERFORMING ORGANIZATION Department of Microbiology and Immunology	6b OFFICE SYMBOL (If applicable) NA
7a NAME OF MONITORING ORGANIZATION Office of Naval Research	
6c ADDRESS (City, State, and ZIP Code) Medical College of Pennsylvania 3300 Henry Avenue Philadelphia, PA	7b ADDRESS (City, State, and ZIP Code) 800 N. Quincy Street Arlington, VA 22217-5000
8a NAME OF FUNDING/SPONSORING ORGANIZATION Office of Naval Research	8b OFFICE SYMBOL (If applicable) ONR
9 PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER N00014-82-K-0669/P00004	
8c ADDRESS (City, State, and ZIP Code) 800 N. Quincy Street Arlington, VA 22217-5000	10 SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO: 61153N; PROJECT NO: RR04108; TASK NO: NR666-008; WORK UNIT ACCESSION NO:

11 TITLE (Include Security Classification)
Mechanisms of Rapid Nonspecific Resistence Induced by Immunomodulators: Delineation Using Selective Depletion of Cells

12 PERSONAL AUTHOR(S)
Morahan, Page S.; Volkman, Alvin

13a TYPE OF REPORT	13b TIME COVERED	14 DATE OF REPORT (Year, Month, Day)	15 PAGE COUNT
Final	FROM 8/86 TO 7/87	January 28, 1988	46

16 SUPPLEMENTARY NOTATION

17 COSATI CODES			18 SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	Macrophages, monocytes, PMN, NK cells, HSV-2, EMC, Listeria, immunotherapy, interferons, immunomodulators, C. parvum, MVE-2, lipoidal amines, ^{89}Sr, continued...
08			

19 ABSTRACT (Continue on reverse if necessary and identify by block number)

The effects of a wide variety of endogenous and exogenous immunomodulators were applied in mouse models permitting in vivo and in vitro analysis of effector mechanisms in rapid non-specific resistance. Major attention was given to models in which circulating monocytes and NK cells were depleted in short-term and long-term administration of the bone-seeking isotope, ^{89}Sr. The data support the concept that resistance to Listeria monocytogenes, and EMC and HSV-2 virus is independent of normal levels of circulating monocytes and NK cells. Monocyte depletion with ^{89}Sr was associated with loss of a C. parvum inducible population of splenic MØ but had no detectable effect on resident peritoneal MØ. Absence of peritoneal MØ elicitation in ^{89}Sr mice dictated experiments to deplete tissue MØ to further assess their contribution to resistance. Techniques for the use of free and liposome-encapsulated cytotoxins were studied in vitro and in vivo. Toxins included ricin, ricin A-chain and dichloromethylene diphosphonate (DMDP). Extensive tissue MØ depletion cont'd.

20 DISTRIBUTION/AVAILABILITY OF ABSTRACT	21 ABSTRACT SECURITY CLASSIFICATION
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS	(U)

22a NAME OF RESPONSIBLE INDIVIDUAL	22b TELEPHONE (Include Area Code)	22c OFFICE SYMBOL
Dr. J.A. Majde	(202) 696-4055	ONR

DD Form 1473, JUN 86 Previous editions are obsolete SECURITY CLASSIFICATION OF THIS PAGE

cont'd.

18. Subject terms: bone marrow depletion, liposomes, prostaglandins, ricin, ricin A-chain, dichloromethylene diphosphonate.

19. Abstract:

... in vivo was achievable with ricin and ricin-liposomes. Resistance to Listeria infection is impaired in ricin-liposome treated mice with increased microbial growth despite significant depletion of peritoneal MØ under these conditions. DMDP-liposomes IP gave erratic results. DMDP liposomes IV, however, yielded more promising short-term results with effective depletion of liver and spleen MØ. The IP liposome toxin models are too complicated to permit adequate evaluation but features such as apparent restoration of blood monocyte levels and dose-related elicitation/depression of PMN suggest that detailed analyses in refined protocols could yield useful information.

END

DATE

FILMED

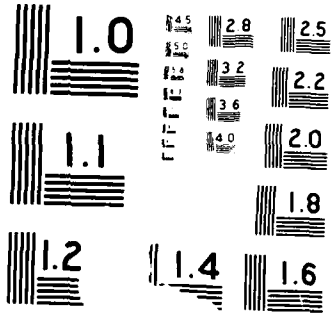
5-88

DTIC


Cork

AD-A191 438 MECHANISMS OF RAPID NONSPECIFIC RESISTANCE INDUCED BY IMMUNOMODULATORS D (U) MEDICAL COLL OF PENNSYLVANIA PHILADELPHIA DEPT OF MICROBIOLOG P S MORAHAN ET AL 2/2

UNCLASSIFIED 28 JAN 88 N00014-82-K-0669 F/G 6/5 NL


1.0
1.1
1.2
1.4
1.6
1.8
2.0
2.2
2.5
2.8
3.2
3.6
4.0
4.5
5.0

SUPPLEMENTARY

INFORMATION

AD #1914-3C

Errata for:

Final Report

"Mechanisms of Rapid Nonspecific Resistance Induced by Immunomodulators: Delineation Using Selective Depletion of Cells"

Office of Naval Research Contract N00014-82-K-0669/~~P00004~~

Principal Investigator: Page S. Morahan
The Medical College of Pennsylvania
Philadelphia, PA 19129

Co-principal Investigator: Alvin Volkman
East Carolina University Medical School
East Greenville, NC

p. 10 - last line, change to "efffective in 89Sr mice"

p. 11 - line 4, change "89Sr" to "88Sr"
p. 12 - line 6, add "in normal mice." at end of sentence, and delete the next sentence (last sentence of that paragraph).

p. 23 - add the following publication.

Pinto, AJ, D Stewart, G Jendrasiak, A Volkman, N van Rooijen and PS Morahan. Selective depletion of macrophages using toxins encapsulated in liposomes: effects on antimicrobial resistance. Eds.: I Fidler and G Lopez-Berestein. In: Liposomes in the Therapy of Infectious Diseases and Cancer, UCLA Symposia on Molecular and Cellular Biology (Alan R. Liss, NY), in press 1988.

p. 27 - Figure legend 14a-c,

line 5. Change "after Listeria" to "before Listeria".
last word. Change "not Listeria" to "not 89Sr".

END
DATE
FILMED
7-88
DTIC